The Role of the Interferon System in Hepatitis-C Virus Replication

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Contents

Abstract

The Hepatitis-C virus is the leading cause of chronic hepatitis. The host immune system identifies specific viral patterns, and induces an Interferon response. IFNs are known to suppress HCV replication *in vitro*, by the induction of antiviral responses mediated by Interferon stimulated genes (ISGs) and the current treatment regimen of IFN-α and Ribavirin has proven partly successful.

However, HCV remains a poor inducer of interferon. This feature is attributed to the ability of the HCV proteins to cleave[1,2] or successfully circumvent[3] most proteins involved in the interferon pathway. IFN-independent antiviral mechanisms have been shown to exist as bypass mechanisms in the event of viral evasion of the IFN system.

In this study, gene knock-out lines lacking key molecules involved in induction, reaction and amplification of type I IFNs as well as antiviral responses induced by alternative pathways was used to determine the role of the type I IFN system in the restriction of HCV replication. The data provided here indicate the novel antiviral functions of Interferon regulatory factors (IRFs) and interferon stimulated genes (ISGs) in limiting HCV replication in mouse cells. Mouse cells with a competent interferon signalling system did not permit replication of HCV whereas cells with individual defects in the type I interferon response, IRF-3, STAT-1 and the ISG PKR permitted detectable levels of replication. However, replication was observed in cells with defective IRF-5, IRF-7 and IRF-1 only in the absence of a type I interferon response.

These results argue, that lesion of these genes weaken such IFN-independent defences to the extent that HCV replication is detectable upon neutralization of secreted IFN. Therefore, we conclude that in WT MEFs, IFN-dependent and independent mechanisms contribute to the control of HCV replication, and that IFN-independent defences are mediated through IRF-5, IRF-7, IRF-1 and PKR. Interestingly, IFNAR$^{-/-}$, IRF-3$^{-/-}$ and STAT1$^{-/-}$ cells showed no further increase in replication of the subgenoimc replicon upon type I IFN depletion which could be based on lower expression levels of IRFs[4,5]. This is compatible with the current view that these factors are essential for the IFN-mediated antiviral activity. Taken together, these data show that apart from the predominant type I IFN response, IFN-independent antiviral effects are involved in restricting HCV replication in mouse fibroblasts.

Similarly, data from primary mouse hepatocytes indicate that in addition to the type I interferon responses, other interferons such as type III interferon may also exert antiviral effects.

1. Introduction

The hepatitis-C virus (HCV) affects approximately 200 million individuals worldwide. It was in 1990 that Houghton and colleagues had identified the causative agent of the elusive non-A non-B hepatitis (NANBH) to be a characteristically and pathologically different virus. This virus was then termed as the 'Hepatitis-C' virus. Today, decades after its identification, HCV related morbidity and mortality has risen to pandemic proportions. Although current treatment regimes of pegylated IFN and ribavirin has proven largely successful, not all treated patients attain viral clearance. Therefore, the need for a suitable vaccine and more effective treatment measures are pressing.

1.1 Overview of Hepatitis-C virus

Non-A, Non-B viral hepatitis was first identified in 1975 through serological studies that tested negative for Hepatitis-A and Hepatitis-B viruses[6]. Prior to its identification and classification, NANBH was known to be the etiologic agent of transfusion-derived hepatitis, associated frequently with significant morbidity and mortality[7]. The first viral sequences of the pathogen were identified in 1989 by screening lambda phage complementary DNA expression libraries developed from nucleic acids extracted from NANBH infected chimpanzees and screening them against serum derived from a NANBH patient [8]. This causative agent of the Non-A, Non-B viral hepatitis regarded to cause serious illness was discovered to be an RNA virus of approximately 9.6 kb and renamed Hepatitis-C virus (HCV). Since its discovery, scientific research on HCV has come a long way. Known to cause both acute and chronic hepatitis, with the latter often leading to fibrosis, cirrhosis and hepatocellular carcinoma, Hepatitis-C infection has been recognized as a global health concern affecting nearly 3% of the world population.

1.1.1 Taxonomy and Genotypes

HCV has been classified as the sole member of the genus *Hepacivirus* within the *Flaviviridae* family.

Differences in the genetic variability of HCV occur at several levels. Firstly, owing to the highly heterogeneous genome of the Hepatitis C virus, it is classified into 7 different genotypes [9]. HCV genotypes 1 and 2 are relatively globally distributed whereas genotype 3 is prevalent in South East Asia and India; Genotype 4 is found in the African continent and

in the middle East ; Genotype 5 is common to South Africa and genotype 6 is predominant in South East Asia [10,11]. Finally, in an attempt to adapt to the host and evade the immune system, HCV diverges significantly over time within an infected individual giving rise to several 'quasispecies'.

Genotypes 1a and 1b are relatively more resistant to treatment with IFN-α as compared to genotypes 2a, 2b and 3a. Since treatment response has been shown to be associated with viral genotype, the identification of the genotype helps determine treatment regimen and dose and predict treatment outcome [12].

1.1.2 Prevalence and Risk factors

An estimated 170-300 million people are infected with Hepatitis-C [13]. However, the extent of disease transmission on a global scale is not well established, because acute infection is generally asymptomatic associated with mild flu-like symptoms making many infections going unaccounted for and also because estimates from the developing world are largely variable or not available [14].

Today, the major cohort of HCV infected individuals belong to the injecting drug user (IDU) category. Other common sources of infection include percutaneous exposure to blood through cosmetic procedures and cultural practices like tattooing, acupuncture, body piercing, circumcision etc [15]. Nosocomial transmission of the virus is possible and includes needle-stick injuries among health care workers, infection during surgery or dental treatment, and other medical procedures[15,16].

Since diagnosis is largely elusive and treatment outcome is variable, the need for vaccine development is pressing.

1.1.3 Pathogenesis and clinical manifestations

One of the salient features of HCV infection is its propensity to establish a persistent infection ultimately leading to hepatic disease. Pathogenesis differs between acute and chronic Hepatitis-C [17,18]. The incubation period for acute HCV infection is from 2-10 weeks, with an average incubation phase of six to seven weeks[19-22]. 60-70% of patients infected with acute HCV are asymptomatic and resolve infection; 20-30% present with jaundice; and 10-20% have nonspecific symptoms such as loss of appetite, fatigue, nausea, weight loss and abdominal pain or discomfort [23,24]. Elevations in liver enzyme levels are analyzed to assess the extent of hepatic injury. Due to the late onset of anti-HCV antibodies, and non-apparent symptoms, acute Hepatitis C often goes unrecognized. Of all

the infected patients, approximately 80% progress to chronic hepatitis. The persistence and detection of HCV RNA 6 months after the acute phase is said to be characteristic of chronic hepatitis.

1.1.4 Standard mode of therapy

Acute hepatitis-C is resolved if asymptomatic or cured in most cases if diagnosed[25,26], whereas treatment of chronic hepatitis-C is largely variable, effective in only half the patient population. Therapy of chronic hepatitis-C has evolved from IFN alpha (IFNα) monotherapy to the use of a polyethylene glycol modified form, called pegylated IFNα (pegIFNα) with increased biological half-life, administered together with the nucleoside analogue ribavirin. This combination therapy has been the standard mode of treatment since 2001 ensuing in the best case, a sustained virological response (SVR) rate of 46–55%[27-29]. Due to the added advantage of an extended half life, patients need be administered IFN alpha only once a week as compared to unmodified IFN alpha.

The aim of therapy is sustained virological response as assessed by PCR for serum viral load (VL) after 4 and 12 weeks of therapy.

Recent developments in the understanding of HCV, particularly in its biology have enabled us to appreciate the interest in antiviral strategies directed against specific proteins of the virus. DAAs (Direct Acting Antiviral) are inhibitory molecules directed against proteins that have important enzymatic or structural functions in virus propagation. Protease inhibitors boceprevir and telaprevir have been FDA approved for treatment when administered in combination with peg-IFN-α and ribavirin. Many other drugs are currently in various phases of pre-clinical trials.

The lack of fidelity of the RNA dependent RNA polymerase (RdRp) with respect to its proof reading capacity combined with high replication rates [30] results in the generation of a large number of quasi-species. This pool of variants might naturally comprise strains that are more resistant to drugs. Additionally, under specific drug pressure, the risk of selecting for a dominant resistant strain is high [31] and is therefore considered a matter of grave concern. The ideal drug must therefore decrease viral load but maintain an increased barrier for resistance, and be effective pan-genotypic, and across isolates and escape mutants.

1.1.5 Vaccines

Currently, there exists no available vaccine to prevent HCV infection. The development of a vaccine against HCV is challenging owing to the presence of diverse genotypes and quasispecies expressing epitope heterogeneity [32]. However, spontaneous clearance of the virus, the development of neutralizing antibodies and adaptive responses against the virus in infected humans[33,34] lend hope to the development of an effective vaccine.

Hence, further research aiming at identifying a target viral protein that can be used in vaccines is warranted. In parallel, due to the large inter- individual variation in response to HCV infection, studies on host responses to infection are required.

1.1.6 Viral tropism of HCV

HCV typically infects hepatocytes but data also confirm detection of HCV RNA in B lymphocytes as well as in the CNS. HCV primarily infects hepatocytes and the ensuing cellular insult results in various pathological conditions characteristic to HCV such as elevated AST/ALT and bilirubin levels. This eventually leads to severe irreversible conditions such as fibrosis, cirrhosis, and HCC.

Although HCV has been regarded as hepatotropic, various reports have detected HCV RNA in PBMCs as well as in the CNS. Various immune defects as well as lymphomas observed in patients chronically infected with HCV have been correlated to the infection of PBMCs.

Although the presence of HCV has been detected in PBMCs and is hypothesized to be the basis of occult infections, whether infection can occur in these reservoirs is still unclear. Similarly, the HCV-associated neuropathogenesis has been attributed to the detection of HCV RNA present in the post mortem brains of HCV infected patients corroborated by the expression of all entry factors required for HCV entry into the brain endothelium[35].

HCV has been shown to replicate in a variety of cell lines of non-hepatic origins such as 293 cells, T [36] and B [37] cells, human brain endothelial cells [35], HeLa cells [38] as well as Human embryonic kidney cells [39]. Also, HCV has been observed to replicate in cells of non-human and non-primate origin such as mouse fibroblasts[40].

1.1.7 The Genome organization of HCV

The HCV genome is a 9.6 kb long single stranded RNA strand. It comprises the genetic codes for the structural as well as the non-structural proteins and is flanked by the two conserved 5' and 3'non translated regions (NTRs). The 5' NTR is essential for viral translation and replication. The 5'NTR region possess the homologous IRES element pivotal in cap-independent translation. The 3' UTR has a tripartite structure and is important for HCV replication. The 3'NTR is a well defined structure comprising a variable region, followed by a poly (U/UC) tract and a conserved 3' X tail sequence.

The HCV polyprotein is processed by both cellular and viral proteases and peptidases to form individual protein sequences. E1 and E2 are envelope proteins highly glycosylated and interact with the host cell entry receptors such as cluster of differentiation 81 (CD81), scavenger receptor class B type I (SR-B1), and occludin (OCLN) mediating cellular entry. The non-structural proteins play important roles in replication, virus processing and have also evolved to antagonize the host response.

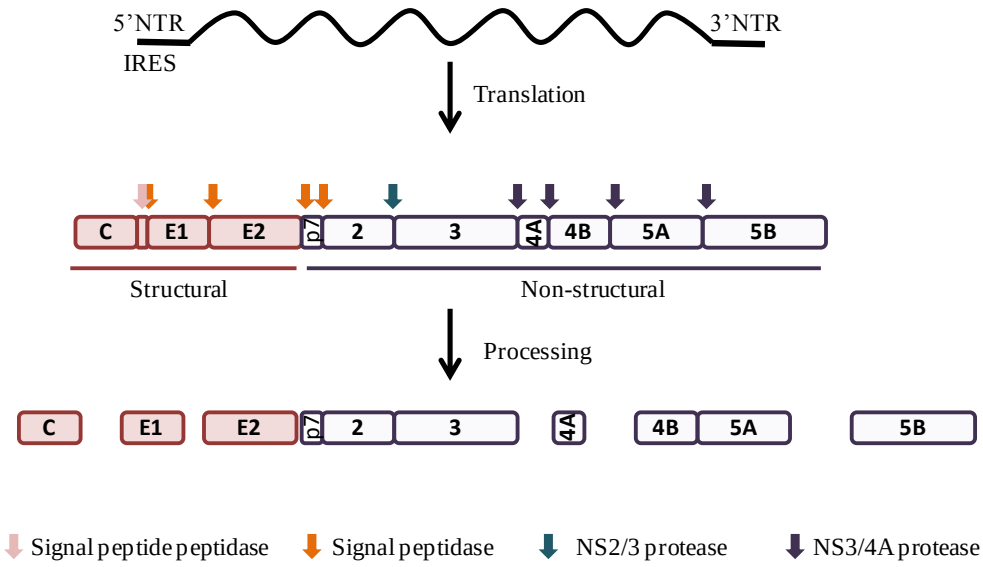

Figure 1: Genome organization and processing of HCV

A schematic representation of the HCV genome. The 5' and 3' NTRs are non translated regions important in replication of HCV, the endogenous IRES element aids in cap-independent translation. The structural proteins E1 and E2 are the envelope proteins whereas the Core protein codes for the capsid structure. Protein p7 is an ion-channel protein. The non-structural elements NS2, NS3 and NS4A are proteases helping in post translation auto-cleavage of the polypeptide. NS4B aids in replication complex formation. NS5A comprises of an ISDR and NS5B is the RNA dependent RNA polymerase. Proteinases and peptidases involved in processing of the polyprotein are indicated by arrows.

The viral protein p7 is an ion channel protein that is indispensable to virus production.
NS2 is also important for virus production but is dispensable for HCV replication. The NS2/3 cyteine protease effectively cleaves the NS2 and the NS3 proteins into fully functional individual proteins. The NS3 protein is a serine protease which in combination with stabilizing factor NS4A cleaves the junctions between itself, NS5A and NS5B. The NS3/4A also effectively cleaves important adaptors of the IFN response such as the MAVS and the TRIF proteins. The NS4B is an integral membrane protein that is localized at the replication complexes called 'membranous web'. These organelles are complexes of non-structural proteins that act as scaffolds for HCV replication. The NS5A has an 'Interferon Sensitivity Determining Region (ISDR)' and mutations in this region are associated with IFN sensitivity that is characteristic to genotypes.
The NS5B is the viral RNA dependent RNA polymerase (RdRp) without which the virus is incapable of replication.

1.1.8 HCV life cycle

The HCV life cycle progresses through multiple distinct stages. Once in the blood stream, the virus travels to the highly vascularized liver.
The initial step of cellular entry is aided by the binding of the E1/E2 envelope glycoproteins to specific receptors on the cell surface. Receptor binding leads to internalization of the clathrin-coated virus particle by endocytosis followed by fusion to an endosomal compartment. The surface receptors required for HCV entry include tight junction proteins like Occludin [41], SR-B1 [42] and Claudin [43] as well as CD81, a tetraspanin family protein [44]. Studies have indicated the role of low density lipoprotein receptor (LDLR) [45,46], DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin) as well as C-type lectin domain family 4 member as receptors for HCV through their interaction with E2. Fusion of the clathrin-coated vesicle to the endosome results in acidification inducing the release of the single stranded, positive sense viral RNA into the cytoplasm of the infected cell. Once released, the viral RNA serves as a template for further replication as well as cap-independent translation. The translation machinery of the HCV genome employs the HCV-IRES [47] element coded by the 5' non-translated region. The HCV IRES directly binds the 40S ribosomal subunits and subsequently

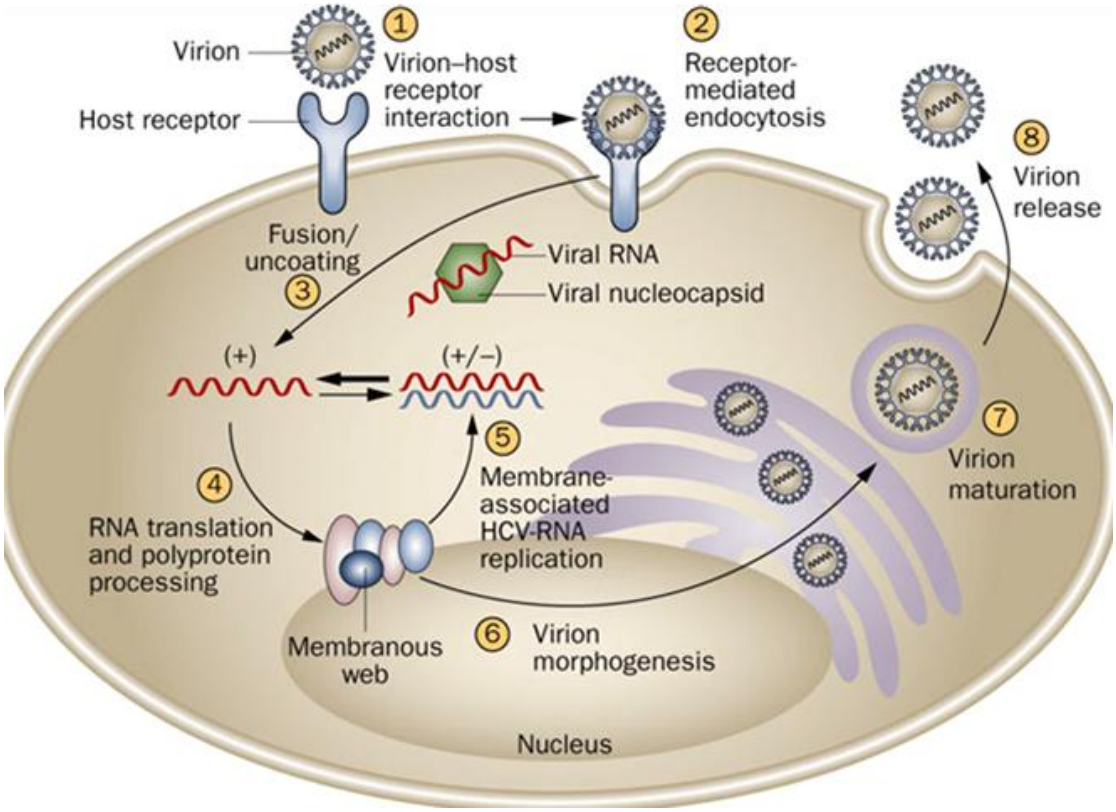

Figure 2 : The HCV life cycle

The HCV infects a cell through 1) interactions between the glycoprotein on their surface with the tight junction and other receptors on the cell membrane. 2) the virus is endocytosed into the cell following which 3) it uncoats and releases its genetic material. Here, the viral RNA undergoes 4) translation and 5) replication. 6-8) RNA processing, cellular trafficking and maturation of the virus particle finally results in virion release [48].

recruits eukaryotic initiation factor (eIF) 3 followed by the ternary complex of Met-tRNA–eIF2–GTP to form a 48S intermediate, before forming an active 80S complex. Following translation, the polypeptides undergo processing by host signal peptide peptidases. Soon after translation, the replication complex comprising the viral genome, host and viral proteins are formed. Replication is initiated at specific membrane-derived organelles called 'membranous webs' [49] where the RNA dependent RNA polymerase begins replicating the viral genome. Since the RNA dependent RNA polymerase (RdRp) is devoid of proof reading activity, the resulting progeny are error-prone. The RNA thus replicated are either used as templates for further replication, translation or are simply packaged into capsids forming virions that are exocytosed and now capable of infection. The lack of fidelity of the RdRp gives rise to several variants of the virus termed 'quasi-species'. The continuous

turn-over of variant strains, although not always 'fit' allows for the selection of strains with epitopes capable of evading immune responses.

1.2 The Type I IFN Response

The type I IFNs are induced in response to viral infections. The viruses are recognized as non-host due to specific pathogen associated molecular patterns (PAMP). The receptors that sense these 'signatures' exhibit organelle specific localization. Some of these receptors are discussed below.

1.2.1 Viral sensors

RLRs

Although the cell membrane and the endosomal vesicles are guarded against pathogen attack by TLRs, certain pathogens can uncoat themselves in the cellular cytoplasm escaping detection by the TLRs. This situation is avoided by the presence of specific cytoplasmic sensors that recognize pathogen signatures. One such receptor is the family of RIG-I-like receptors (RLRs) that constitute RIG-I, MDA-5 and the negative regulator LGP-2.

RIG-I and MDA5 consist of a DexD/H box RNA helicase domain, two N-terminal caspase-recruitment domains (CARDs), and a C-terminal repressor domain all of which are important in a strictly regulated signalling process. Unlike RIG-I and MDA-5, LGP-2 has no CARD domain and acts in regulating the RIG-I/MDA-5 signalling process. RIG-I and MDA-5 although similar in their location and functions have relatively distinct substrate specifications. RIG-I recognizes ssRNA of defined lengths along with 5' triphosphates terminal regions on mRNAs, resulting from viral replication, or from RNaseL mediated cleavage products of the virus and also by artificially introduced products of *in vitro* transcription[50,51]. The MDA-5 recognizes comparatively long poly (I:C) regions and viral genomic dsRNA. Apart from the differences in genetic elements, the receptors also recognize different viruses[52]. Where RIG-I recognizes paramyxoviruses, MDA-5 is important in recognition of picornavirus. Although similar in their action, RIG-I recognizes Japanese Encephalitis virus (JEV) and HCV whereas West Nile virus and Dengue virus; albeit belonging to the *Flaviviridae* family are both recognized by MDA-5[53].

TLRs

Cellular surveillance on the membrane and the endosomal compartments are carried out by TLRs. TLRs are proteins containing an extracellular domain of leucine-rich repeats (LRRs) and a cytoplasmic TIR (Toll/IL-1R homology) domain [54]. 13 TLRs have been recognized in humans. The reason for their diversity and differential localization is to enable the cell to detect a wide array of pathogens differing in structural as well as genetic composition. TLRs localized on the cell membrane (TLRs 1, 2, 4, 5 and 6) protect against extracellular pathogens whereas TLRs on the endosomal compartments (TLRs 3, 7/8 and 9) help defend against pathogens that are taken up within the cell. TLRs 2, 3, 4, 7, 8, and 9 have been reported to be important in the detection of viral components[55]. TLR2 and TLR4 are best understood in the context of recognizing Gram-positive (lipoteichoic acid) and Gram-negative bacteria (lipopolysaccharide) respectively, but are also important viral sensors. In the case of TLR2, the difference in the outcome of viral or bacterial detection lies in the internalization of the receptor post recognition. Upon detection of a virus, the TLR2 is immediately internalized leading to the activation of the NFκB dependent inflammatory pathway as well as the IFN dependent antiviral pathway whereas in a bacterial attack only the inflammatory response is activated.

TLR3 is activated by dsRNA (dsRNA virus or replicative intermediates) and its synthetic surrogate polyinosinic:polycytidylic acid (polyI:C). The endosomally localized TLR7/8 is known to recognize ssRNA as well as guanosine- and uridine-rich ribonucleotides. TLR9 recognizes the (cytidine-phosphate-guanosine) (CpG) motifs on the pathogen genome and is therefore important in the detection of DNA viruses such as HSV[56].

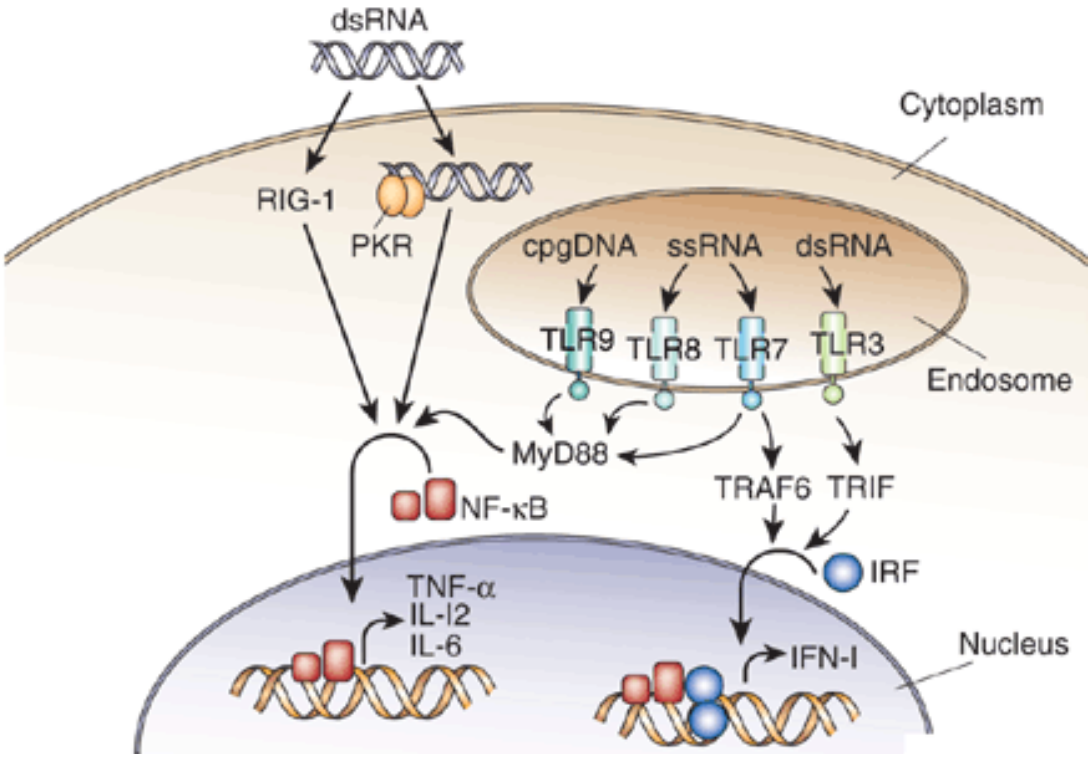

Figure 3: Signalling pathways of pattern recognition receptors

Cytosolic RIG-I as well as the endosomal TLRs play an important role in recognizing specific pathogen patterns. This results in the downstream activation of cellular adaptor molecules such as MyD88 and TRIF. The activation is relayed to the nucleus and leads to the induction of an interferon and an inflammatory response.

1.2.2 Signalling of Viral Sensors

The induction of an immune as well as an inflammatory response is the result of a series of synchronized cascades initiated by the detection of a pathogen eventually leading to the induction of IFN stimulated genes and proinflammatory cytokines. Since pathogens can enter cellular cytoplasm through endosomal vesicles, pattern recognition receptors are specifically distributed within a cell. This organelle specific localization enables detection of a wide range of pathogen signatures. Of the Pathogen Recognition Receptors (PRRs), the most extensively studied are the Toll-like receptors (TLR). The TLRs are Type 1 transmembrane proteins signal between endosomes and the cellular cytoplasm. TLRs are located on the plasma membrane as well as in the endosomal vesicles. This localization enables efficient detection of viral particles present in the extracellular matrix as well as the recognition of viral genome released within the endosomes during viral uncoating.

Once the virus is released into the cytoplasm, they are patrolled by the RIG-I like receptors (RLR family), the nucleotide oligomerization domain-like receptors, as well as DNA sensors such as the members of the AIM2 family.

RLR Signalling

Signalling through RIG-I like receptor depends on the CARD-CARD interaction between the receptor and the mitochondrial antiviral signalling [MAVS] (also known as CARD adaptor inducing IFN-β [Cardif], mitochondria-located adaptor molecule IPS-1 and virus-induced signalling adaptor [VISA]). Upon recognition of specific pathogen signatures, RIG-I/ MDA5 undergo conformational changes that permit close interaction with the CARD domain of MAVS. This interaction leads to the assembly of proteins on the mitochondrial surface that subsequently triggers the induction of downstream proteins. This complex activates TBK1 and IKKα/β that phosphorylates IRF-3 and IRF-7, and induces an IFN response. Additionally, it also activates the kinase activity of the IKKγ complex which results in NF-κB activation.

TLR Signalling

The primary role of TLR molecules is to recognize pathogen signatures and induce host responses against them. To do so, they require adaptor proteins that relay information from the localized TLRs into the nucleus. The adaptors that TLRs use are the myeloid differentiation factor 88 (MyD88), MyD88-adaptor-like (Mal), TIR domain-containing adaptor inducing IFN-β (TRIF), and TRIF-related adaptor molecule (TRAM). The use of one or more of these adaptors leads to the induction of a distinct and specific immune response. TLR3 activates downstream targets through adaptor protein TRIF whereas TLR7/8 and TLR9 induce antiviral responses specifically through MyD88. TLR2 has been shown to signal through MyD88 via Mal and TLR4 can signal through MyD88 as well as through TRIF.

1.2.3 Induction of Type I IFNs

The IFNs are critical mediators of an antiviral response. The IFNs, comprising type I (IFN-α (alpha), IFN-β (beta), IFN-κ (kappa), IFN-δ (delta), IFN-ε (epsilon), IFN-τ (tau), IFN-ω (omega), type II (IFN-γ), and type III (IFN-λ 1,2 and 3), play a crucial role in the host

immune response. The type I IFNs are induced during an infection. These pleiotropic cytokines act in an autocrine and paracrine manner to activate host cell antiviral responses and also alert the surrounding cells of the viral invasion. As described previously, upon detection of specific pathogen signatures (PAMPs) by Patter Recognition Receptors PRRs, transcription factors like IRF-3 are activated. Transcriptional activity of IRF-3 is induced by virus and dsRNA-stimulated, C-terminal phosphorylation. The activated IRF-3 translocates to the nucleus and together with NF-κB, AP-1, and the nuclear architectural protein HMG-I(Y) (high mobility group protein [non histone chromosomal] isoform I and Y) assemble into an enhanceosome complex on the IFN-β promoter. This results in recruitment of specific molecules, such as the co-activators CBP and/or p300 to initiate transcription and synthesis of IFN-β [57-59].

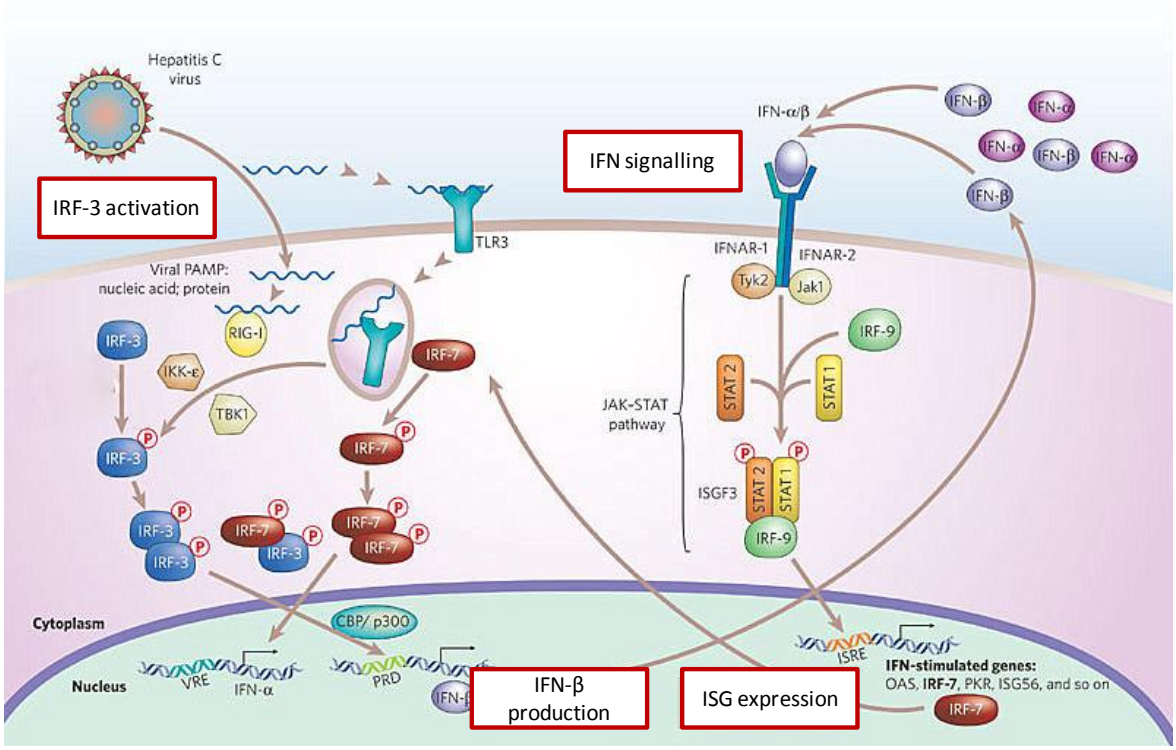

Figure 4: Type I IFN production and signalling

Detection of pathogen by pathogen recognition receptors (TLRs, RIG-I) leads to the activation of downstream adaptor molecules eventually leading to the activation of IFN and ISGs. IRFs are factors that relay activation signals from the cytoplasmic or endosomal PRRs to the nucleus. IFN-α/β bind to the IFNAR receptor which leads to the activation of the Janus kinases Tyk2 and Jak1 which activates STAT1 and STAT2 proteins. STAT1 and STAT2 form a complex with IRF-9 called the ISGF3 which binds to ISRE elements of ISG e.g. IRF-7. IRF-7 is activated and induces IFNαs which leads to the amplification of the IFN response.
Adapted from Gale et al. Nature 2005

IRF-3 displays constitutive expression in the cytoplasm in most cell types, whereas IRF-7 is only expressed upon induction by IFNs, an expression pattern that is also largely cell type specific[60]. Therefore, activation of IRF-3 allows instant production of IFN-β upon viral invasion omitting the need for de novo synthesis. The further amplification of this loop depends on the production of IFN-β and the subsequent binding to the IFN-α/β receptor. This second loop requires activation of transcription factors such as IRF-9 and other molecules such as the (Signal Transducers and Activators of Transcription) STATs[61]. In addition, both IRF-3 and IRF-7 possess the potential to either homodimerize or heterodimerize with each other, permitting activation of distinct IFN genes[61,62].

The preferential activation of the *ifn-β* promoter by IRF-3 is due to its limiting DNA binding potential [62]. In comparison, IRF-7 has a broader DNA binding specificity and is capable of inducing both IFN-β and IFN-α efficiently, thereby contributing to the amplification of the primary IFN response. Thus, the differential gene expression patterns induced by IFN-α/β is attributed to the differential expression and binding specificity of the IRFs.

1.2.4 Antiviral Signalling of Type I IFNs

IFN induction leads to specific autocrine and paracrine binding of IFN to its receptor resulting in stimulation of a wide array of genes essential for antiviral defense. These steps are orchestrated by strict and complex mechanisms. Upon IFN receptor activation, downstream Janus kinase (JAK) and Tyrosine Kinase (TYK2) phosphorylate STAT proteins at specific serine and tyrosine residues. Thus activated, the STAT proteins can now assemble into a complex along with IRF-9 and actively translocate into the nucleus. Once in the nucleus, this complex called the (Interferon stimulated gene factor 3) ISGF3 binds to the promoters of the ISGs thereby transcribing them. ISG activation can also result from the binding of STAT1 homodimers to the IFN-γ activated sites (GAS) elements. The induction and activation of antiviral proteins renders the cell capable of limiting viral spread and elimination of virus-infected cells.

In hepatocytes, double-stranded RNA (dsRNA) triggers two independent pathways of host defense through retinoic acid-inducible gene I (RIG-I) and Toll-like receptor 3 (TLR-3) [63]. The recognition of Pathogen Associated Molecular Patterns (PAMP) results in the rapid induction of IFN-alpha and IFN-beta and subsequent activation of intracellular signalling

events leading to expression of IFN-stimulated genes (ISGs) that are central to antiviral responses[64].

Expression profiles of IFN-stimulated genes obtained from livers of patients with Chronic Hepatitis-C as well as chimpanzees with experimental acute Hepatitis-C were shown to be upregulated [65,66]. However, elevated ISG levels are not an indication of chronic infection as some chronically infected patients show little to no upregulation [66], despite comparable levels and duration of virus infection.

IRF3 (IFN regulatory factor 3), a highly regulated transcription factor constitutively expressed in the cytoplasm in a latent inactive form, plays a key role in regulating the synthesis of IFN-β (17).

Some of these antiviral proteins include Protein Kinase R (PKR), 2',5'oligoadenylate synthase (2',5'-OAS), myxovirus-resistance proteins (Mx), ISG15, ISG56, TRIM79 alpha and dsRNA-dependent adenosine deaminase (ADAR).

The RNA-dependent protein kinase R (PKR), a serine/threonine kinase is stimulated during viral invasion. PKR, when activated upon dsRNA binding of viral genomes or replication intermediates gets autophosphorylated after which it subsequently phosphorylates the eukaryotic translation initiation factor-2 (eIF-2).

The role of eIF-2 in the initiation of peptide synthesis in mammals is directed to deliver Met-tRNAi to the 40S ribosome. eIF-2 composed of three subunits (α, β, and γ) binds to the Met-tRNAi in a GTP-dependent manner to form a ternary complex which attaches to the 40S subunit of the ribosome. Upon delivery, eIF-2 is released from the initiation complex. eIF-2 activity is autoregulated by phosphorylation of the α subunit at position S51. eIF-2α phosphorylation, results in an exaggerated affinity of eIF-2 for eIF-2B leading to competitive inhibition of eIF-2B and an immediate arrest of translation initiation [67]. Similarly, 2',5'-OAS, another IFN-inducible gene makes use of a cellular endonuclease RNaseL. Upon activation of 2'5'-OAS by dsRNA or replication intermediates ATP gets converted to an adenosine oligomer 2'5'-A which then configures latent RNaseL to its active form[68]. Cleavage products of this endonuclease can also trigger RIG-I [69] thus further increasing the activity of RNase L.

1.2.5 Antiviral Signalling of type III IFNs

Type I IFNs are important in directing antiviral immunity. However, another family of molecules was identified that had properties similar to type 1 IFNs but were structurally

and genetically distinct. These IFNs were referred to as the type III IFNs or broadly IFN-λs. Unlike IFN-α which is expressed by all nucleated cells, response to IFN-λ seems to be restricted to epithelial cells. Tissues rich in epithelial content such as intestines, lungs and skin were found to be more responsive to IFN-λ. Evolutionarily, IFN-λ is said to have been evolved to protect mucosal and epithelial cells from pathogen insult. Structurally, the IFN-λ family of cytokines is similar to the Il-10 receptor family. There are 3different genes that encode 3 different forms of IFN-λ: IFN-λ1, IFN-λ2 and IFN-λ3.

The triggers for IFN-λ are similar to that of the type 1 IFN and largely stimulus dependent. The type III IFN receptor is composed of the IL-10Rβ subunit and the IL28Rα that are important for signal transduction. IFN-λ receptor activation leads to the activation of STAT-1 and STAT-2 molecules that complex with IRF-9/p48 forming the ISGF3 complex. This induces the transcription of several hundred IFN stimulated genes. The ISGs induced by type III IFN was shown to be similar to that induced by the type 1 IFNs. Therefore, similar to type1 IFN, type III IFNs have been shown to possess antiviral, anti-proliferative and immune modulatory functions. A recent study on chimpanzees has described the dominant role of IFN-lambda over type 1 IFN in the induction of downstream ISGs in the hepatocyte microenvironment [70].

1.2.6 Virus host interactions

The drastic variations in HCV pathogenesis is largely inter-individual dependent. These variations occur at several levels during the recognition and interaction of the virus by the host which ultimately defines disease outcome. Upon infection of the liver, IFNs are produced that result in the induction of an antiviral state aiding in limiting HCV replication [71]. Therefore, it is not surprising that HCV has evolved several strategies to evade the IFN system [1,2]. In the liver, the innate immune system after having recognized the virus also directs the adaptive immune response. The Kupffer cells of the liver play an important role in pathogen clearance and also recruit NK cells and T cells to the site of infection [72-74]. While NK cells produce IFN-gamma (IFN-γ) to limit viral replication, activated DCs present antigens and produce type 1 IFNs aiding in restricting HCV replication. However, the specific roles of the cellular immune response in HCV infection are largely elusive. The role of T cells in viral pathogenesis is relatively well established[75]. During the acute phase, the effector function of CD8+ T cells and the helper CD4+ T cells in the liver and the peripheral blood serve in clearing the virus. The importance of these two cell types is

further corroborated by increased viremia observed in experimentally infected chimpanzees with depleted T cells. However, during chronic infections the CD4+ T cell responses were absent and the CD8+ T cell responses were observed to be dampened. The reasons for T cell dysfunction have been attributed to the expression of programmed death 1 (PD-1); a T cell inhibitory molecule, to the suppression of T regs [76], and the increased secretion of interleukin (IL)-10 [77].

1.2.7 Evasion of the host immune system by HCV

Pathogen entry into cells is recognized by the cellular host factors that recognize and stimulate an anti-microbial response. When HCV infects hepatocytes, several of its pathogen signatures are recognized by the pattern recognition receptors (PRR). The 3'NTR of the HCV genome harbours a poly(U/UC) tract which in addition to the 5'-PPP region is reported to be recognized by the RIG-I receptor. Similarly, the TLR3 recognizes dsRNA formed as a replication intermediate. Activation of PRRs leads to the induction of antiviral as well as proinflammatory cellular responses.

HCV like most viruses has evolved mechanisms to evade such an immune attack. Several protein components of HCV have evolved to actively counteract key regulatory molecules in the innate immune signalling pathway. One such viral component is the NS3/4A viral protease that cleaves adaptor proteins TRIF and MAVS thereby stunting the downstream TLR3 and RIG-I signalling respectively. Inhibition of NS3 function is observed to restore RIG-I signalling. The two recently approved antiviral drugs Boceprevir and Telaprevir both target the NS3 protease. The induction of (suppressor of cytokine signalling) SOCS3 is upregulated leading to compromised IFN signalling. Similarly, HCV replication induces protein phosphatase 2A (PP2A), which results in increased binding of STAT1 to its inhibitor PIAS1 (protein inhibitor of activated STAT1). Apart from the inhibitory effects exerted directly by the HCV proteins, HCV has also evolved strategies to indirectly alter the immune response. The upregulation of USP18 is said to interact with IFNAR and inhibit the subsequent JAK/STAT signalling, it is corroborated by evidence of increased USP18 in livers of patients with chronic HCV. Increased host protein kinase PKR, results in phosphorylation of the eukaryotic initiation factor eIF-2α that suppresses cellular translation thereby blunting the antiviral response. This mechanism leaves the HCV translation machinery unaffected as the virus translation can proceed through IRES mediated ribosome scanning.

Taken together, HCV has evolved not only its genome to directly restrict HCV replication mechanisms but has also altered the host response pathways to aid in increased HCV replication.

1.3 HCV model systems

1.3.1 Cell culture models

The establishment of experimental model systems for HCV has been practically challenging. Although the HCV genome has been readily available since 1989, cell culture systems as experimental models were successfully developed only much later. Till date, the chimpanzee remains the only animal model susceptible to HCV infection although small rodents and dogs as model systems are emerging.

Various cell culture systems for efficient replication and infection have been developed, the first ever reported case of HCV replication being in 1999 with the discovery of the subgenomic replicon system[78]. The subgenomic replicon is a bicistronic RNA in which sections of the HCV genome encoding the structural and part of the nonstructural proteins were replaced by the neomycin phosphotransferase (NPT) gene driven by the homologous IRES in the 5'UTR, and a second heterologous EMCV-IRES (from Encephalomyocarditis Virus) translating the non-structural proteins NS3–5B.

Since then, the replicon system has been modified in ways that have allowed for the insertion of variations in the marker cassettes, the addition of self-cleaving ribozyme [79] chimeric replicon systems J6JFH1[80] etc. The system has been widely used for analysis of HCV RNA and proteins, biochemical and structural characterization of the viral replication complex, for high throughput screening for drug discovery, and determination of antiviral resistance.

The Con1 (encoding genotype 1b) replicon system was a major breakthrough, until it was discovered that the strain resisted virus production in cell culture and productive replication in chimpanzees. It was hypothesized that either the cells lacked the required machinery to drive virus production or that the acquired mutation that enhanced replication potential had rendered the clone incapable of forming infectious virions in culture.[81].

This unexpected setback was soon overcome by the discovery of the JFH1 strain obtained from a patient with Japanese Fulminant Hepatitis (JFH1). The JFH1 strain had an inherent replication potential manifold higher than the Con1 strain and replicated in hepatic as well as non-hepatic cell lines without the need for adaptive mutations[38,82]. This discovery then

became the basis of infectious particle production in cell culture wherein *in vitro* transcribed RNA transfected into Huh7 cells gave rise to infectious HCV cell culture particles referred to as HCV cc.

The replicon system has since then been expanded to include virus production systems from genotypes 1a (H77) and 1b (Con1) as well as various reporter systems that have been established for microscopic analysis[83,84].

1.3.2 Animal models

The chimpanzee is currently the only animal model susceptible to HCV infection. Although several of the advancements in the field of HCV from its discovery to the host response to the virus is attributed largely to chimpanzee studies, the animal model is expensive, and is restricted due to ethical constraints and limited accessibility. Advancements in rodent models have led to the development of several models such as immunotolerant rat models with transplanted human liver cells and the HCV trimera model wherein irradiated mice were reconstituted with bone marrow cells from severe combined immunodeficient (SCID) mouse and transplanted with HCV-infected liver cells[85]. However, these models suffer due to low viremia, limiting their use in drug efficacy studies.

Another significant advancement in the development of a mouse model for HCV propagation was the SCID/albumin-urokinase type plasminogen activator (Alb-uPA) [86] mouse. This mouse model is based on a SCID background transplanted with normal human hepatocytes. It alleviates several disadvantages plaguing models that had been developed before. With reduced liver toxicity owing to the expression of the Alb-uPA transgene and its immunocompromised background, the SCID/Alb-uPA mouse enables generation of high titres of virus production upon inoculation of HCV patient serum. However, due to the immune compromised background, the elucidation of the immune responses against the virus is impossible.

Difficulties encountered in culturing HCV in cell lines of varied origins strongly suggests the involvement of several viral and host cellular factors in the replication and completion of the HCV life cycle. Deciphering the host factors required for the completion of the HCV life cycle in non-primate and non-hepatic cells is important in unravelling the biology of HCV, and also in providing a basis for a smaller, more suitable animal model. Therefore,

studies aimed at understanding host factors with respect to HCV infection and replication is strongly warranted.

1.4 Biosynthesis of MicroRNA

MicroRNAs are short RNA sequences that aid in post-transcriptional regulation. They are ~22 nucleotide sequences that silence gene expression by binding to complementary sequences in the 3' NTR of target mRNAs.

The synthesis and processing of microRNAs involves 3 important steps. After the synthesis of the primary transcript (pri-miRNA) in the nucleus aided by the RNA polymerase II, the pri-miRNA undergoes a round of partial processing generating the precursor form, pre-miRNA. The processing into the precursor form is mediated by the ribonuclease III enzyme Drosha and the nuclear protein DiGeorge Syndrome Critical Region 8 (DGCR8) also referred to as 'Pasha' in invertebrates. Upon translocation to the cytoplasm, pre-miRNA is subjected to an additional round of processing mediated by ribonuclease III enzyme Dicer complex generating the mature and functional duplex miRNA. The mature strand consists of two segments; a 'guide' strand complementary to the target mRNA and a 'passenger' strand that is usually degraded. The guide strand complexes with Argonaute 2 protein to form the RNA-induced silencing complex (RISC). Once loading on the RISC complex, the miRNA is transported to the 3'NTR of the target mRNA where it can either cleaves it or blocks translation.

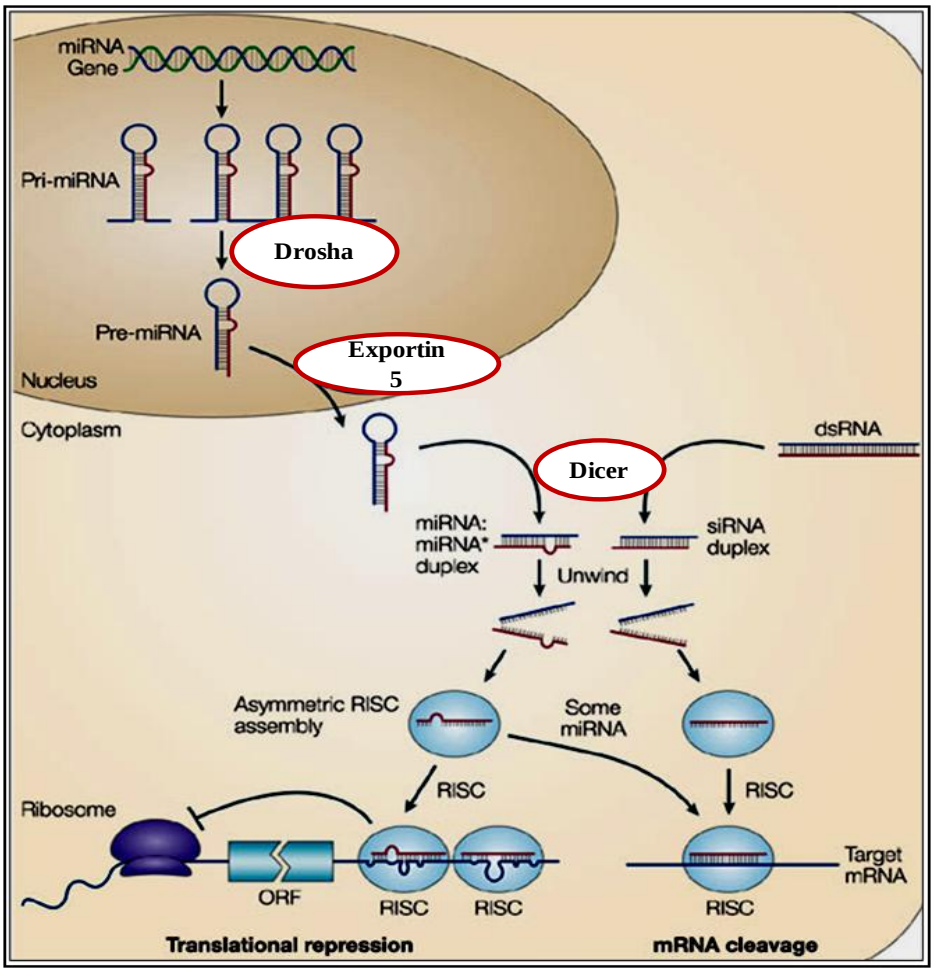

Figure 3: Biosynthesis of MicroRNA

The synthesis of microRNAs requires three important steps. The synthesis of the primary transcript (pri-miRNA) in the nucleus is aided by the RNA polymerase II. This is then partially processed to form pre-miRNA by the ribonuclease III enzyme Drosha. Upon translocation to the cytoplasm by exportin proteins, pre-miRNA is processed by the Dicer complex to generate the mature and functional duplex miRNA. It is then loaded on the RISC complex, enabling it to either cleave the target mRNA or block translation.[87]

1.4.1 MicroRNA-122 and HCV

Micro RNAs are endogenous non-coding RNAs that are important transcriptional regulators leading to translation repression and gene silencing by degrading mRNA[88,89]. Originally identified in *C.elegans*, miRNAs have now been identified to be critical in process such as fatty acid metabolism, cell proliferation and apoptosis[90,91]. However, the exact mode of regulation remains largely elusive. MiR-122 is specifically expressed in the liver and is thought to be important for regulating tissue specific gene expression profiles [92].

Making up for 70% of the miRNA in the adult liver, miR-122 has been observed to play an important role in fatty acid metabolism and cholesterol biogenesis [93,94]. Notably, miR-122 has been identified as a host factor crucial for efficient replication and production of HCV [35,95]. MicroRNA-122 interacts with two defined sites of the 5'NTR [95] which are highly conserved between genotypes 1 and 2a and this assembly has been shown to aid in regulating viral replication and translation[96]. The exact effect of miR-122 on hepatitis-C replication is still unclear. While some reports suggest the involvement of miR-122 in the accumulation of HCV RNA regulating the rate of amplification, some others suggest a direct role on viral translation due to increased association of the ribosome with the HCV RNA. In accordance to this, the binding of miR-122 to the 5'NTR is said to relieve the structural conformation of the viral IRES element to aid in translation. Although the exact mechanism remains to be elucidated, the importance of miR-122 in HCV propagation has been highlighted by recent therapeutic targeting of miR-122 using an antisense inhibitor as an approach to decrease HCV viremia [97]. Ectopic expression of MiR-122 has also been shown to rescue replication levels in non-hepatic cell lines indicating its role in determining viral tropism[39]. Also, reduced levels of MiR-122 in patients with chronic Hepatitis-C are associated with poor response to IFN therapy[98].

1.5 Research objectives

The interferon system leads to the restriction of HCV replication *in vitro*. Consequently, Interferons are successfully used in therapy but not all patients achieve the desired sustained virological response. It is unknown whether and to what extent, IFNs contribute to the restriction of HCV in the mouse.

Improvement of treatment outcome requires the analysis of HCV restriction by the interferon system. Therefore, there is a need to understand the IFN-dependent as well as independent mechanisms in detail. Additionally, in a clinical setting where patients develop auto-antibodies against IFNs, the individual antiviral properties exerted by the IRFs might be critical in determining disease outcome.

This study, will aim at identifying the impact of IFN-dependent and IFN-independent mechanisms on the restriction of HCV replication. Embryonic fibroblasts and hepatocytes isolated from mice lacking key molecules involved in induction, reaction and amplification of type I IFNs will be used to determine their ability to maintain HCV replication.

HCV is recognized by the host system which induces an interferon response against it. Liver biopsies of chronic HCV-infected patients exhibit increased expression levels of ISGs[99]. The data could be validated in experimentally infected chimpanzees[65,100] and revealed that the outcome of infection could be determined by analyzing the ISGs induced at very early time points. To this end, a stable cell line expressing an inducible HCV replicon should be established that permits the detection of early host responses i.e. ISG expression immediately following recognition of pathogens by the cellular system.

2. Results

2.1 Chapter 1: The Role of IRFs in limiting HCV replication

Hepatitis-C has been recognized as an emerging public health concern and the current treatment regimen of pegylated Interferon alpha in combination with ribavirin has proven only partly successful.

Achievement of sustained virological response is variable and is dependent not only on the genotype of the virus but also on the infected host. Host factors such as IL28 gene polymorphism have been attributed to the clearance of HCV[101-104]. Since viral clearance is largely inter-individual dependent even within the same genotype, it is evident that the host immune system has an important role in determining disease outcome. Therefore, the aim of this thesis was to identify the role of the interferon system in the clearance of HCV. The Hepatitis-C demonstrates strict species and tissue tropism. The species barrier operates at different levels within a cell. Firstly, HCV tropism has been attributed largely to the strict requirement for entry receptors [41] found on the cell surface of humans and chimpanzees but not in small rodents making infection models naturally impossible on murine backgrounds. Secondly, post entry; the virus can be blocked at various stages of its replication. This could be a result of specific cellular restriction factors that are either constitutively expressed or triggered upon viral invasion or due to the absence of certain complementary factors that aid in competent replication.

In order to determine the function of the interferon system in limiting HCV replication, embryonic fibroblasts derived from mice knocked out for various effectors of the interferon system were used.

2.1.1 Isolation and Conditional immortalization of mouse embryonic fibroblasts

Embryonic fibroblasts were isolated from pregnant mice 13 days post coitus (dpc). At this stage of gestation, the red organs are coalesced and are easy to excise enabling isolation of fibroblasts. Freshly isolated mouse embryonic fibroblasts were conditionally immortalized using third generation self-inactivating lentiviral vector carrying the proto-oncogene large T antigen from Simian Virus 40 (SV40) under the control of a conditional tetracycline-dependent promoter. Expression of the immortalization cassette depends on the presence of

tetracycline[105]. The expression of the immortalizing cassette is carried out by the reverse transactivator (Tet-on system) in a bicistronic fashion. Doxycycline administration leads to activation of the reverse transactivator activating the positive feedback loop. Therefore, supplementing the cell culture media with Doxycycline, (a derivative of Tetracycline) leads to the activation of the Tet-dependent promoter which results in the continuous transcription of the immortalizing cassette. Cells transduced with lentivirus coding for the immortalization cassette are therefore 'conditionally immortalized'.

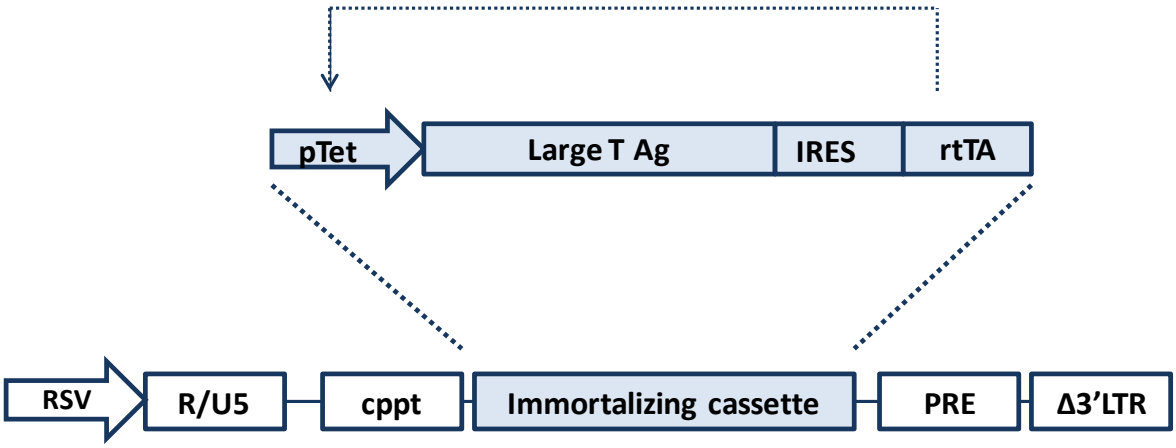

Figure 4 : Schematic representation of the immortalizing cassette.

The lentiviral construct coding for immortalizing cassette containing the Large T antigen which is driven under the control of a tet promoter followed by the reverse transactivator connected by an IRES element. The entire cassette is inserted into a third generation self-inactivating lentivirus[106]. Adapted from: T.May *et al* 2007.

This system enables the continued passage of cells in the presence of permissive conditions; the absence of which (without doxycycline) restricts proliferation potential enabling cells to attain a 'primary-like' phenotype [106].

2.1.2 Expression of miR-122 in mouse embryonic fibroblasts

One of the hallmarks of Hepatitis-C biology is its requirement for specific cellular factors essential for replication and propagation. The liver specific micro RNA, miR-122 has been identified as a positive enhancer of Hepatitis-C viral replication in cells of hepatic as well as non-hepatic origin[39,40,96]. In this study, specific miR122 sequences were cloned within sequences derived from mir-30[107] downstream of a spleen foci forming virus promoter

(SFFV). The second cistron consisted of an encephalomyocarditis virus internal ribosomal entry site (EI); a green fluorescent protein (GFP); and a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE). The miR30 sequences flanking miR-122 enables precise cleavage of miR-122 and therefore ensures proper functioning[107]. This third generation, self-inactivating lentivirus was used to transduce conditionally immortalized mouse embryonic fibroblasts in order to enhance HCV replication and/or translation. Cells positive for GFP expression were sorted by florescent activated cell sorting.

Figure 5 : The plasmid sequence coding for miR-122.

Schematic representation of the miR-122 expression plasmid. The miR-122 cassette sequence is flanked by miR-30 and is driven by the Spleen Foci Forming Virus (SFFV) promoter. The second cistron includes an Internal Ribosomal Entry Site (IRES) upstream of an eGFP sequence. The woodchuck post transcriptional response element (WPRE) plays an important role in the enhancement of gene expression[108,109].

The resulting sorted GFP^+ population was subsequently positive for miR-122 expression. The supplementation of miR-122 in mouse fibroblasts will aid in enhanced replication of HCV.

2.1.3 MEFs with a competent immune signalling pathway restrict HCV replication

In order to analyze the role of the innate immune pathway in the restriction of HCV replication, the replication of HCV in WT MEFs was studied. Since HCV is species and tissue tropic and displays a strict requirement for entry receptors, infection of mouse fibroblasts which are not natural hosts of the virus is naturally impossible. To circumvent this problem, a widely used surrogate - the subgenomic replicon genotype 2a; JFH-1 (Japanese fulminant hepatitis) was utilized.

The JFH-1 RNA requires little to no adaptation to cell culture and has been shown to replicate in hepatic as well as non-hepatic cells[39,110]. The JFH-1 replicon constitutes genes

coding for both the structural as well as the non-structural proteins of the HCV genome. However, the subgenoimc replicon is designed to code only for the proteins expressing the non-structural genes along with a part of the core protein that is fused to a Firefly luciferase gene functioning as a reporter plasmid. In addition, since the plasmid contains a functional NS5B gene which codes for the viral polymerase, the subgenomic replicon is capable of replicating. Therefore, replication can be monitored by increasing luciferase expression.
Additionally, a modified version of the plasmid with a deletion of three amino acids (GDD) in the NS5B gene coding for the polymerase enzyme (NS5BΔGDD) was utilized as a negative comparison to the polymerase competent stain.

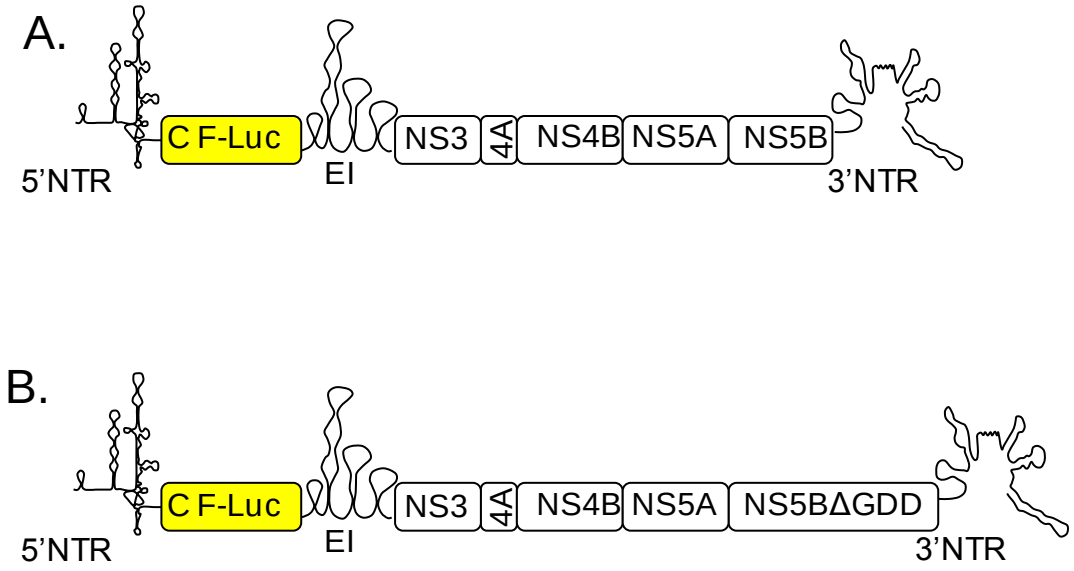

Figure 6 : Schematic representation of HCV subgenomic replicons

A.The HCV subgenomic replicon RNA luciferase reporter (JFH1-Luc) and **B.** the polymerase mutant NS5BΔGDD subgenomic replicon RNA luciferase reporter (JFH1-LucΔGDD). Both subgenomic replicons consists of the 5`NTR, the core N-terminal 12 amino-acid coding sequence fused in frame with the firefly luciferase gene (C F-Luc), the encephalomyocarditis virus internal ribosomal entry site (EI), the NS3-NS5B (the non structural proteins with specific enzymatic functions) coding region and the 3´NTR.

In order to determine the replication potential of the JFH-1 subgenoimc replicon in MEFs with a fully competent interferon system (WT), cells were electroporated with in vitro transcribed RNA derived from JFH-1 and compared to the replication deficient JFH-1ΔGDD subgenomic replicons. The transfected cells were seeded onto 12-well plates in the

absence of Doxycycline. Cell lysates were harvested and assayed for luciferase activity as a read out for replication.

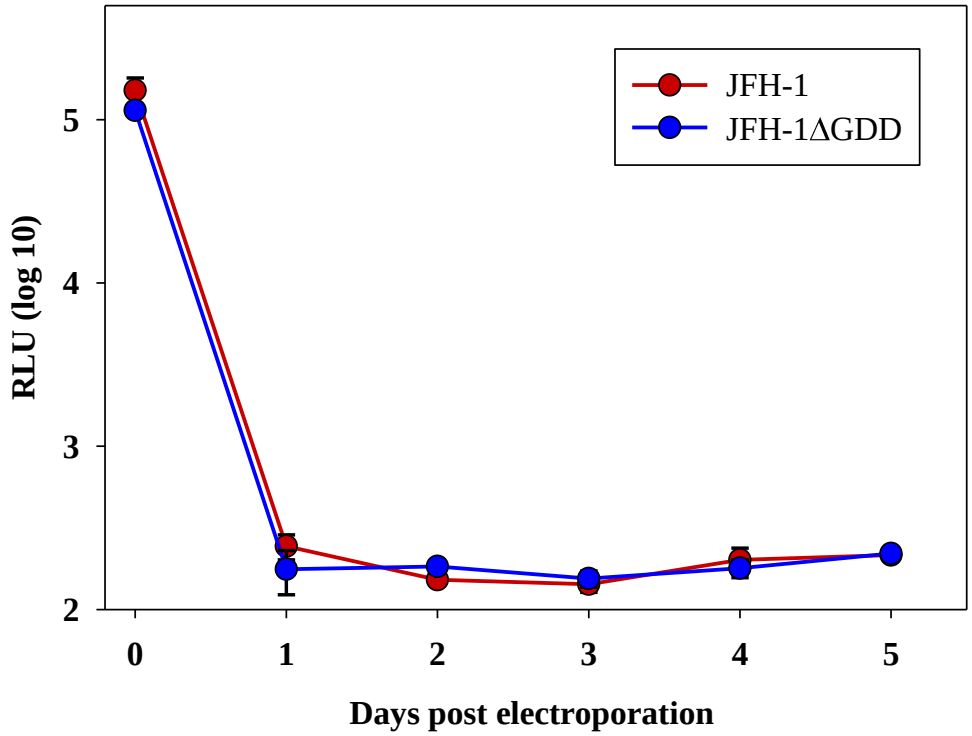

Figure 7 : Transient replication of JFH-1 and JFH-1ΔGDD in WT MEFs

WT MEFs expressing miR-122 were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and each day for the next 5 days.

Luciferase expression at 4 hours post electroporation (Day 0) is a result of translation of transfected RNA and indicates transfection efficiency. However, after Day 0, luciferase expression measured is indicative of an NS5B enabled replicating construct.

As shown in **Figure 7**, luciferase expression in WT MEFs transfected with JFH-1 or JFH-1ΔGDD replicon was indistinguishable up to 5 days post electroporation, suggesting that WT MEFs were unable to maintain detectable HCV replication.

Once HCV has replicated, the replicated strands can act as templates for translation. The resulting polypeptide is co- and post-translationally cleaved by host and viral proteases to yield individual functional proteins. Detection of HCV proteins is a direct measurement of replication. Therefore, the presence of HCV protein NS3 was tested by immunoflourescence.

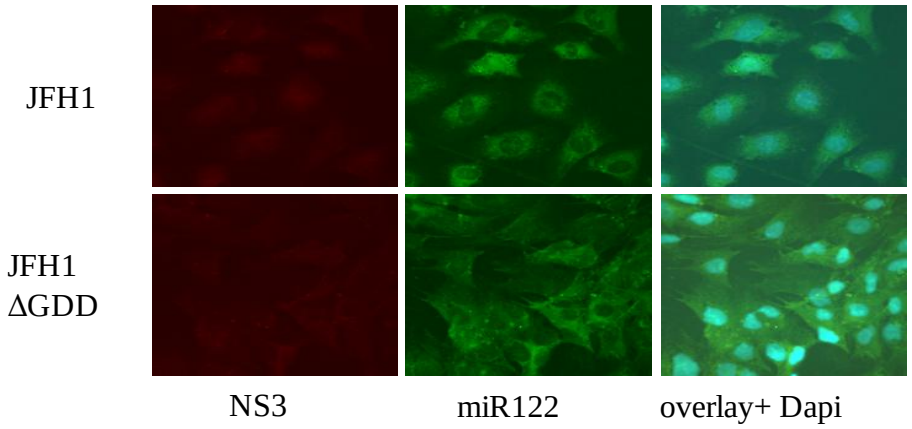

Figure 8 : NS3 protein detection in WT MEFs

WT MEFs stably expressing miR122 (GFP^+) were electroporated with JFH-1 RNA or JFH-1ΔGDD RNA and plated on cover slips. Upon fixation, cells were stained using a primary antibody directed against NS3, followed by a secondary anti-mouse Cy5 conjugated antibody. Coverslips were mounted on Mowiol containing DAPI.

As shown in **Figure 8**, NS3 protein of the HCV subgenomic replicon was not detectable by immunoflourescent staining on day 3 post electroporation in WT MEFs. This indicates that there is no detectable replication and therefore no continued translation of the HCV polyprotein under WT cellular conditions.

Taken together, this suggests the presence of competent virus replication inhibition signals or the absence of additional cellular functions supportive of HCV replication in rodent cells. Additionally, supplementing cells with miR-122 did not lead to detectable HCV replication in WT MEFs.

2.1.4 HCV replicates in IFN receptor deficient MEFs

Since HCV is known to induce an interferon response [111] and replication has been shown to be sensitive to type I interferon, the function of the IFN receptor in limiting HCV replication was tested. A functional type I IFN receptor is important in inducing STAT1 homodimerization and its subsequent binding with IRF-9 leading to the stimulation of interferon stimulated genes and the amplification of interferon induction. This positive amplification loop maintains an effective antiviral response in the cells.

In order to elucidate the requirement of the type 1 IFN receptor in restriction of Hepatitis-C in murine fibroblasts, conditionally immortalized MEFs deficient in the alpha chain of the

type 1 Interferon receptor (IFNAR1) stably expressing miR-122 were utilized. These cells were transfected with the polymerase competent (JFH-1) and deficient (JFH-1ΔGDD) strains of the replicon. The luciferase values were determined 4 hours post electroporation (Day 0) as an indication of transfection efficiency and everyday for the next 5 days.

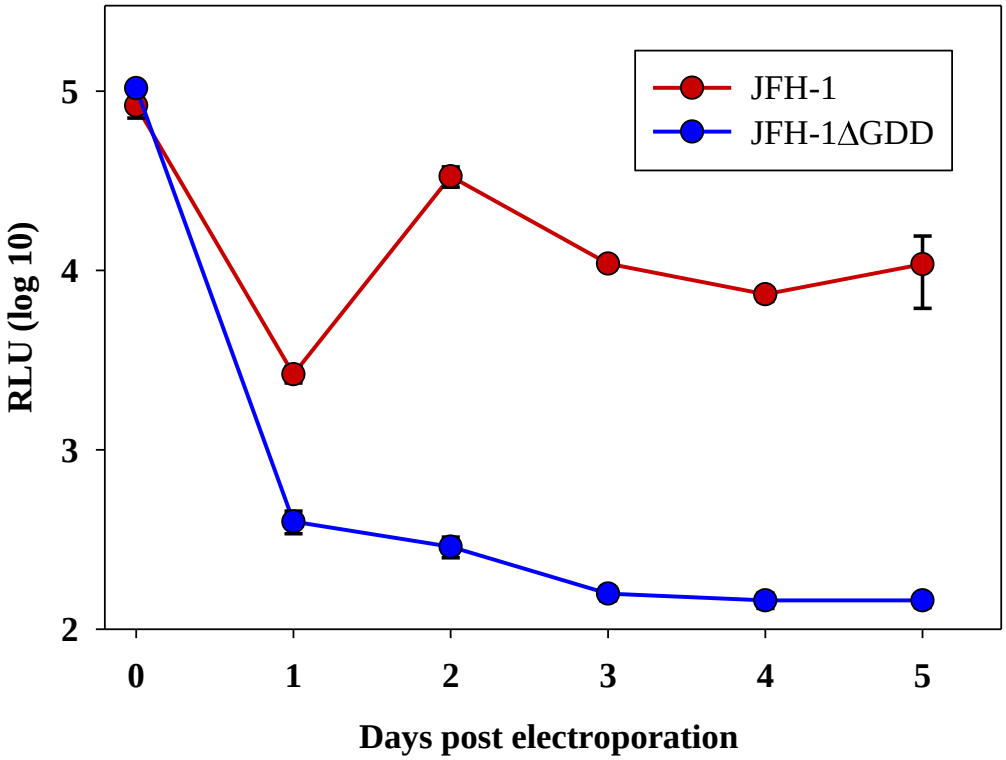

Figure 9 : Transient replication of JFH-1 and JFH-1ΔGDD in IFNAR$^{-/-}$ MEFs

MEFs expressing miR-122 and deficient in Type 1 IFN receptor (IFNAR$^{-/-}$) were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and thereafter for the next 5 days.

Figure 9 indicates that the JFH-1 strain (red line) shows detectable luciferase expression indicative of on-going replication whereas the luciferase expression of the NS5B deficient JFH-1ΔGDD (blue line) strain had dropped below detection limit.

In order to confirm processing of the complete replicon, the presence of HCV protein NS3 was assessed by immunoflourescence staining 3 days post transfection.

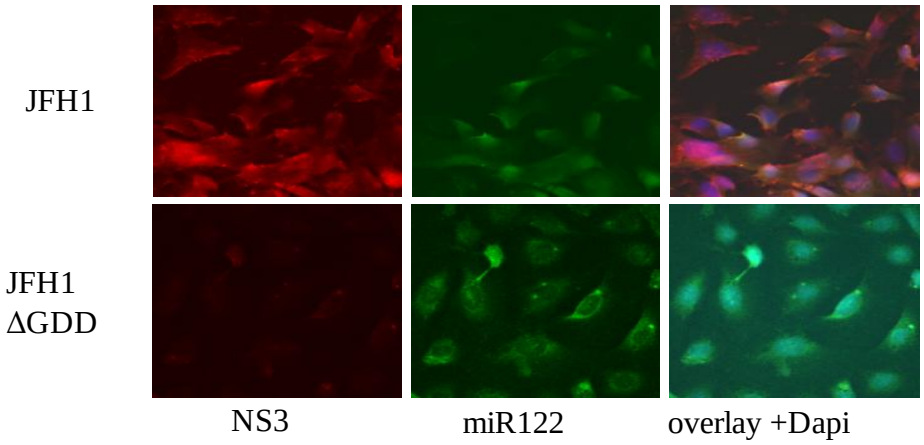

Figure 10 : NS3 protein detection in IFNAR$^{-/-}$ MEFs

IFNAR$^{-/-}$ MEFs stably expressing miR122 (GFP^{+}) were electroporated with JFH-1 RNA or JFH-1ΔGDD RNA and plated on cover slips. Upon fixation, cells were stained using a primary antibody directed against NS3 (red), followed by a secondary anti-mouse Cy5 conjugated antibody. Coverslips were mounted on Mowiol containing DAPI.

As shown in **Figure 10**, the cytoplasmic distribution of HCV protein NS3 in IFNAR$^{-/-}$ MEFs electroporated with JFH-1 RNA is clearly visible in contrast to the cells transfected with the NS5B mutant JFH-1ΔGDD confirming the presence of replicating HCV JFH-1 RNA.

Taken together, these data indicate that the HCV is capable of completing its replication cycle in mouse cells with a defective IFNAR receptor. In addition, immunoflourescence staining of the NS3 protein confirms complete processing of the HCV polyprotein. More importantly, these data indicate that HCV replication in mouse fibroblasts is sensitive to type I IFN; the absence of which enables continued replication.

2.1.5 The role of IRF-3, STAT1 and IRF-7 in HCV replication

With the role of the IFN receptor established as indispensable in limiting Hepatitis-C virus, the focus was to determine additional cellular factors that could limit HCV replication. Interferon regulator factor-3 exhibits cytoplasmic distribution in fibroblasts and is constitutively expressed in a latent, inactive form. This allows for immediate response to

viral infections eliminating the need for *de novo* synthesis, allowing the immediate production of IFN-β.

IRF-3 functions downstream of RIG-I; an indispensible recognition receptor in the identification of HCV and is therefore considered critical to antiviral response. Once activated, IRF-3 translocates to the nucleus and aids in the transcription of IFN-β.

To ascertain the roles of IRF-3 on the replication of HCV, MEFs deficient in IRF-3 stably expressing miR-122 were electroporated with RNA transcribed from JFH-1 or JFH-1ΔGDD subgenoimc replicons. Cells were cultured on 12- well plates and harvested at 4 hours post electroporation (Day 0) and every day for the following 5 days.

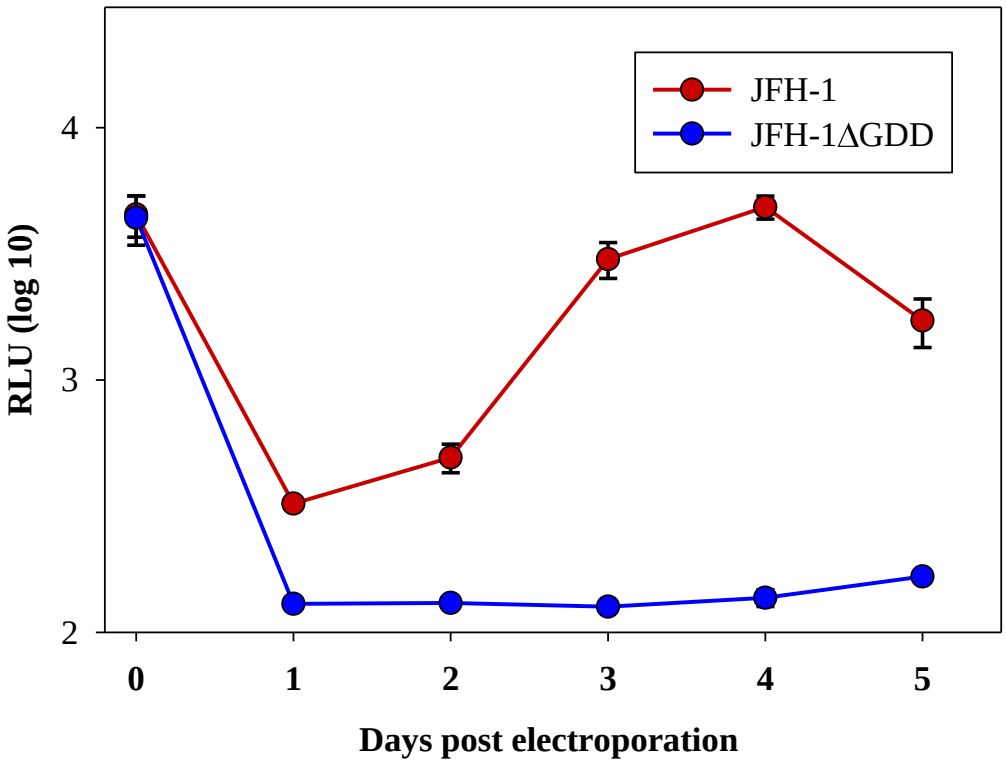

Figure 11 : Transient replication of JFH-1 and JFH-1ΔGDD in IRF-3$^{-/-}$ MEFs

IRF-3$^{-/-}$ MEFs expressing miR-122 were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and everyday for the next 5 days.

In the absence of IRF-3, a sustained replication of polymerase competent HCV (red line) is observed from day 1 post electroporation in the transient reporter assay. This suggests that the RIG-I induced IRF-3 dependent pathway is pivotal in restricting HCV replication. Taken together, these data indicate that in addition to the presence of the type I IFN receptor, IRF-3 is an important factor in limiting HCV replication.

STAT1, a member of the (Signal Transducers and Activators of Transcription) family is an indispensable transcription factor which can dimerize with STAT3 or with itself to bind the GAS (Interferon-Gamma Activated Sequence) promoter element or heterodimerize with STAT2 to bind the ISRE (Interferon Stimulated Response Element) promoter element[112]. Further, given the importance of STAT1 in continued IFN signalling and induction of IFN-αs in establishing an antiviral response, it is not surprising that HCV has evolved to actively counteract this signalling pathway. HCV proteins NS5A [113] and core[3,111] have been shown to interact with STAT1, inhibiting its phosphorylation and degrading it using a proteasome-dependent mechanism.

In order to verify the role of STAT1 proteins in HCV replication inhibition, STAT1$^{-/-}$ MEFs stably expressing mir-122 were transfected with JFH-1 or JFH-1ΔGDD *in vitro* transcribed RNA.

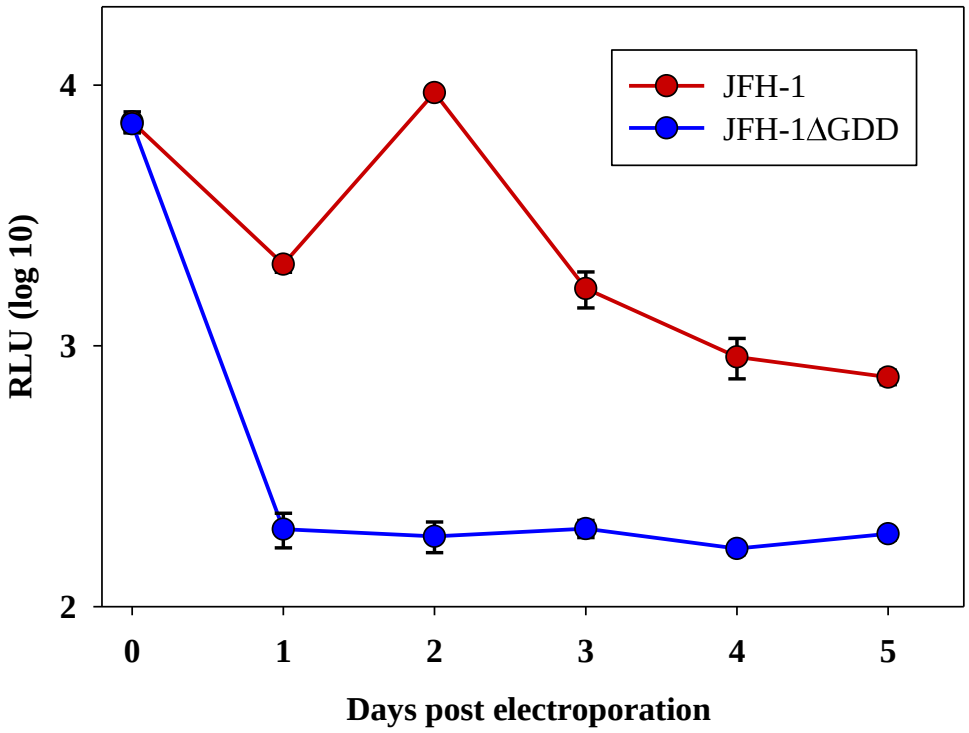

Figure 12 : Transient replication of JFH-1 and JFH-1ΔGDD in STAT1$^{-/-}$ MEFs

STAT1$^{-/-}$ MEFs expressing miR-122 were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and everyday for the next 5 days.

The data shown above indicates that JFH-1 strain was capable of replication whereas the JFH-1ΔGDD was not. The maximum replication potential was observed at day 2 post electroporation following which the luciferase expression dropped consistently but did not reach undetectable levels. This indicates a functional role of STAT1 in limiting HCV replication. These data indicate that the STAT1 pathway is mandatory in limiting HCV replication.

Unlike IRF-3, IRF-7 is induced in response to infection during the amplification process of the IFN loop and is therefore not considered an early response gene. Additionally, IRF-3 and IRF-7 are capable of binding to each other to form heterodimers that translocate to the nucleus to induce differential genes.

Similarly, IRF-7$^{-/-}$ MEFs were electroporated with JFH-1 and JFH-1ΔGDD and the luciferase expression was monitored from cells lysates harvested at 4 hours (D0) post electroporation and every day for the next days.

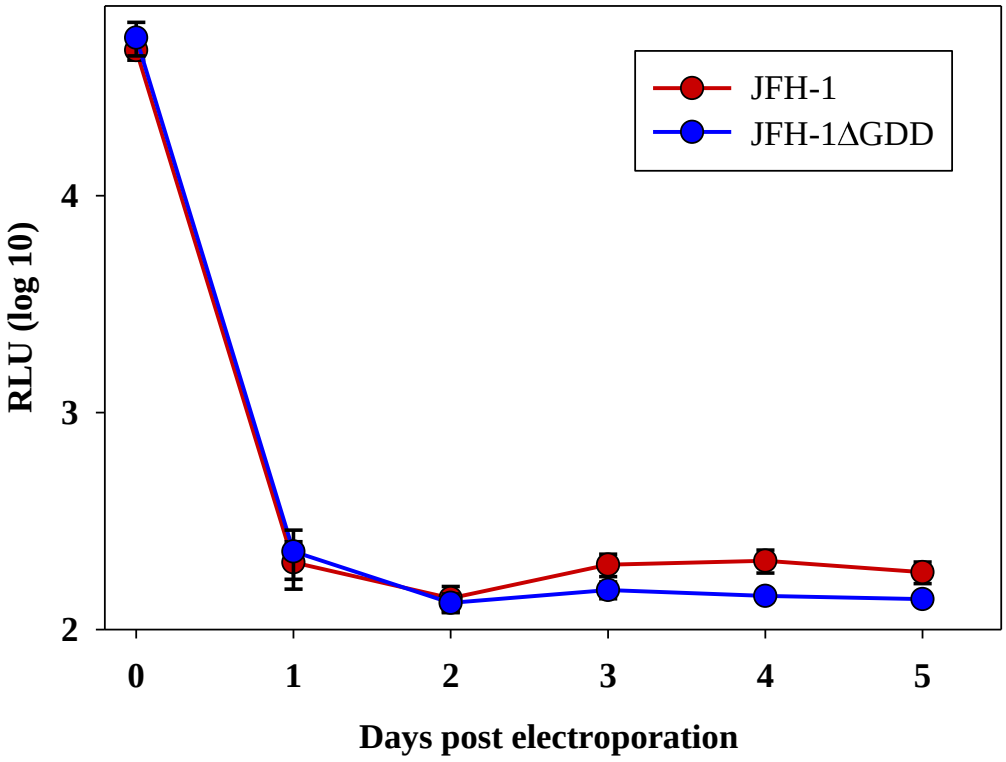

Figure 13 : Transient replication of JFH-1 and JFH-1ΔGDD in IRF-7$^{-/-}$ MEFs

IRF-7$^{-/-}$ MEFs expressing miR-122 were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and everyday for the next 5 days.

The data show no detectable luciferase expression in both JFH-1 and the JFH-1ΔGDD replicons after Day 0. This indicates that the expression levels observed at day 0 were derived from the transfected RNA and that the expression could not be maintained in the following days. This suggests that the JFH-1 replicon was not able to replicate in MEFs in the absence of IRF-7.

To confirm these findings, immunoflourescent staining was performed on electroporated cells 3 days post transfection to determine if the viral NS3 protein was present.

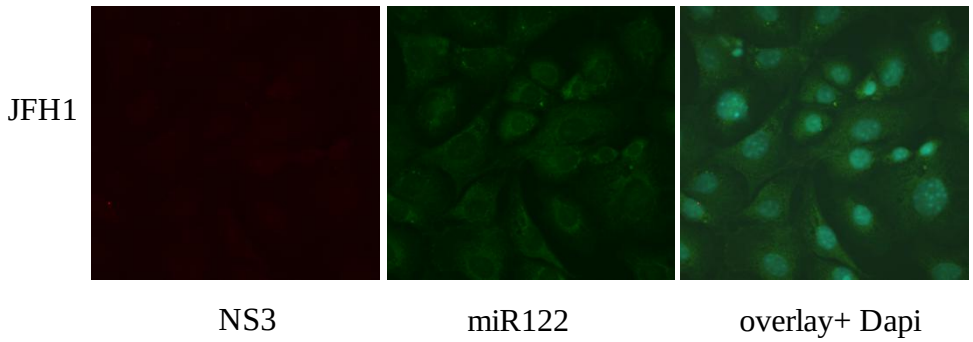

Figure 14 : NS3 protein detection in IRF-7$^{-/-}$ MEFs

IRF-7$^{-/-}$ MEFs stably expressing miR122 (GFP^{+}) were electroporated with JFH-1 RNA and plated on cover slips. Upon fixation, cells were stained using a primary antibody directed against NS3, followed by a secondary anti-mouse Cy5 conjugated antibody. Coverslips were mounted on Mowiol containing DAPI.

In contrast to IRF-3 deficient MEFs, no luciferase expression of the HCV replicon was observed in IRF-7$^{-/-}$ **(Figure 13)** cells after the initial 4 hour peak. This indicates that the HCV subgenomic replicon is not able to replicate. In line with this observation, no NS3 protein was detectable by immunoflourescence. These data indicate that IRF-7 is not essential to restrict HCV replication in MEFs.

Taken together, these results suggest the importance of IRF-3 and the type I interferon receptor in limiting HCV replication in mouse fibroblasts. In contrast, although interferon regulatory factor-7 is required for the amplification of the immune response, it does not play a dominant role in limiting HCV replication.

2.1.6 The role of IRF-5 in HCV replication

The functional relevance of IRF-5, in comparison to the other IRFs is largely unclear. Although a lot is known about the role of IRF-5 with regard to autoimmune disorders, the

exact function of IRF-5 as an antiviral protein is still elusive. Infection with VSV and NDV activate IRF-5 and over-expression of IRF-5 during viral infections has been reported to induce distinct IFNα [114]. The capacity of IRF-5 to heterodimerize with IRF-3 and induce distinct sets of genes might indicate its ability to tailor cellular responses appropriate to specific viruses. In order to ascertain the role of IRF-5 in inhibiting HCV replication, IRF-5$^{-/-}$ mouse fibroblasts stably expressing miR-122 were transfected with RNA transcribed from JFH-1 and JFH-1ΔGDD subgenomic replicons. Luciferase expression was measured at 4 hours post electroporation (Day 0) and everyday for the next 5 days.

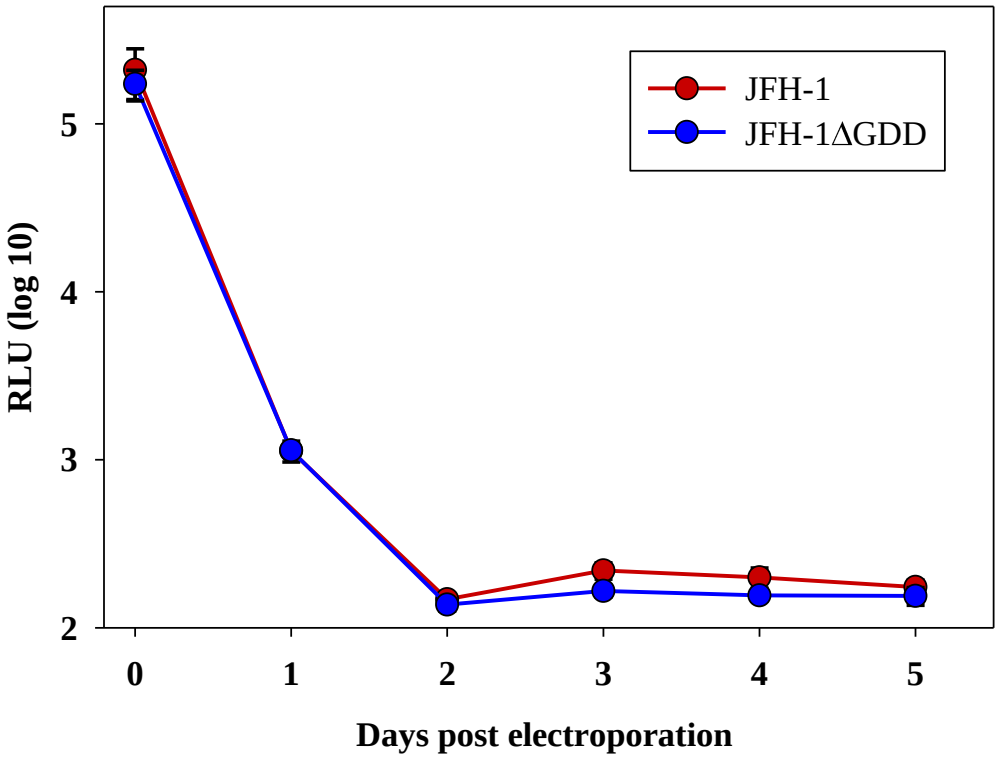

Figure 15 : Transient replication of JFH-1 and JFH-1ΔGDD in IRF-5$^{-/-}$ MEFs

IRF-5$^{-/-}$ MEFs expressing miR-122 were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and everyday for the next 5 days.

As shown in **Figure 15**, the luciferase expression of the JFH-1 was comparable to that of the polymerase deficient strain suggesting its inability to replicate. This indicates that the absence of IRF-5 in mouse fibroblasts does not rescue HCV replication and that IRF-5 has no dominant role in limiting HCV replication.

2.1.7 The role of IRF-1 in HCV replication

The role of IRF-1 in treatment response in patients with HCV has been reported[115,116]. Specific SNP (-300AA) in the promoter regions of IRF-1 have been associated with better treatment outcome[115]. In order to elucidate the function of IRF-1 in the replication of HCV, embryonic fibroblasts derived from mice knocked-out for IRF-1 stably expressing miR-122 were used. RNA transcribed from polymerase competent or polymerase deficient JFH-1 strains were electroporated into IRF-1 knock-out MEFs. Luciferase expression from the subgenomic replicon was measured at 4 hours (Day 0) post electroporation indicative of electroporation efficiency and up to day 5 to detect replication.

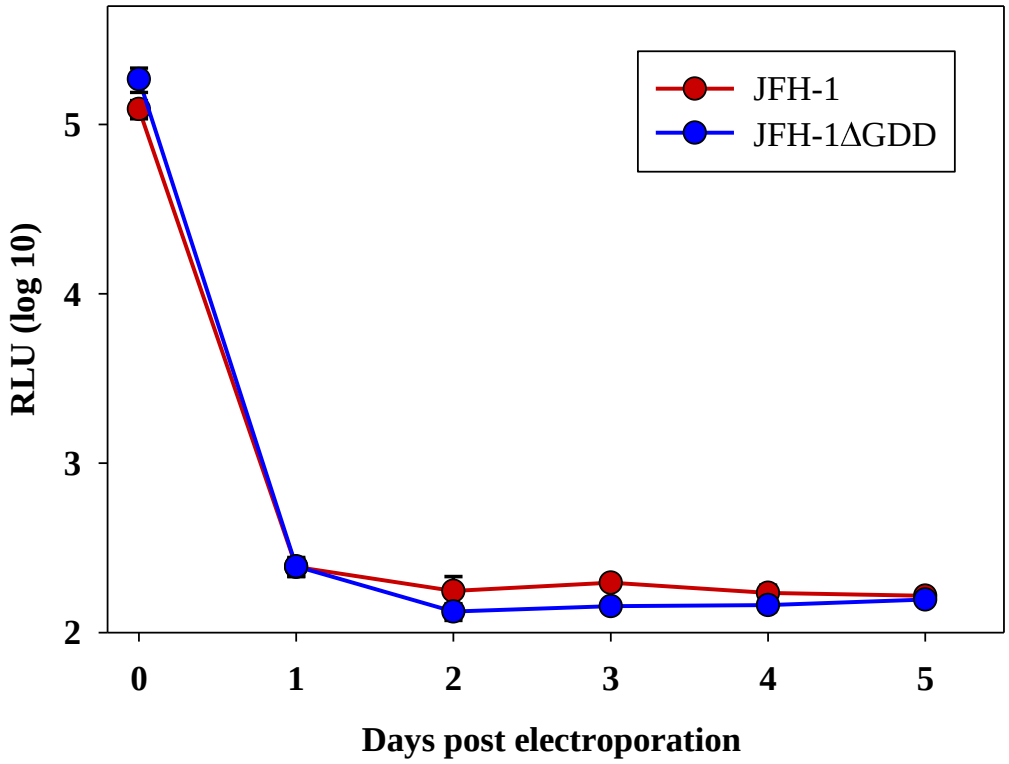

Figure 16 : Transient replication of JFH-1 and JFH-1ΔGDD in IRF-1$^{-/-}$ MEFs

IRF-1$^{-/-}$ MEFs expressing miR-122 were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and everyday for the next 5 days.

As depicted above, the luciferase expression of JFH-1 were comparable to that of JFH-1ΔGDD. Replication of HCV in the absence of IRF-1 was not observed suggesting that IRF-1 was not important in restricting HCV replication.

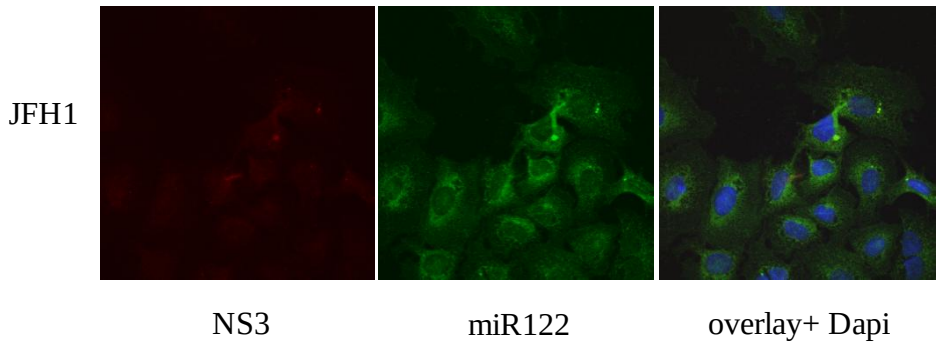

Figure 17 : NS3 protein detection in IRF-1$^{-/-}$ MEFs

IRF-1$^{-/-}$ MEFs stably expressing miR122 (GFP^{+}) were electroporated with JFH-1 RNA and plated on cover slips. Upon fixation, cells were stained using a primary antibody directed against NS3, followed by a secondary anti-mouse Cy5 conjugated antibody. Coverslips were mounted on Mowiol containing DAPI.

Immunoflourescence staining of the NS3 viral protein did not yield any signal suggesting that the IRF-1$^{-/-}$ MEFs could not support viral replication and subsequent translation. This suggests that IRF-1 is not directly involved in the clearance or restriction of HCV.

2.1.8 PKR in HCV replication

PKR (Protein kinase R) belongs to a family of kinases that regulate cellular translation in response to environmental stress. In steady state, PKR is an inactive monomer. It is a serine kinase that is autophosphorylated following binding with dsRNA. Thus activated, phosphorylated PKR further phosphorylates eukaryotic initiation factor eIF-2 inhibiting translation. PKR also phosphorylates IκB, the inhibitory subunit of NFκB.

PKR has been reported as a binding partner of STAT1, where this physical interaction has been described to modulate the transcriptional activity of STAT1 [48]. Studies in PKR-deficient fibroblasts have confirmed that PKR is involved in protection against several virus infections, including HIV-1 [80], HCV [87] , and hepatitis D virus [112].

In order to ascertain the relevance of PKR in HCV replication, MEFs knocked out for PKR and expressing miR-122 were transfected with RNA from JFH-1 or JFH-1ΔGDD. Cells were cultured on 12-well plates and cell lysates were harvested at 4 hours post electroporation (Day 0) and everyday for the following 5 days.

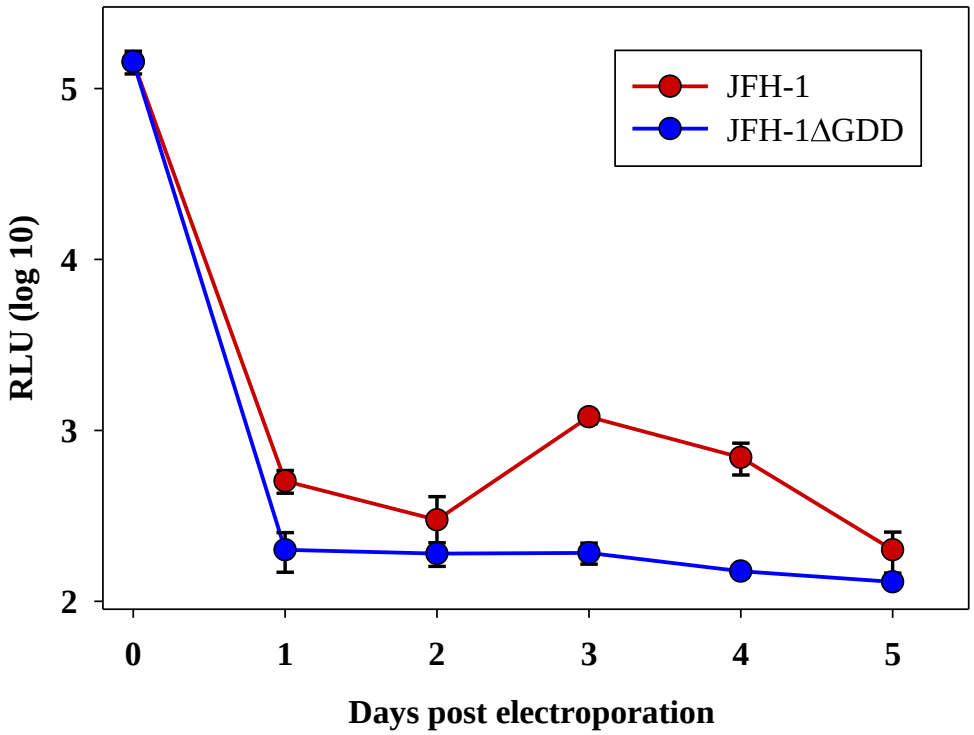

Figure 18 : Transient replication of JFH-1 and JFH-1ΔGDD in PKR$^{-/-}$ MEFs

PKR$^{-/-}$ MEFs expressing miR-122 were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and everyday for the next 5 days.

As shown above, luciferase expression dropped down until day 2 post electroporation after which an increase in replication signals was observed. This suggests a possible inhibitory role of PKR in HCV replication.

2.1.9 MAVS in HCV replication

The Mitochondrial antiviral-signalling protein (MAVS) also referred to as VISA, Cardif or IPS-1 is an important mediator of the RIG-I dependent IFN pathway. Following RIG-I activation, MAVS proteins on the mitochondrial membrane interact with RIG-I through CARD-CARD interactions. This phosphorylates and activates cytosolic IRF-3 and NFκB, which translocates to the nucleus. This leads to the induction of IFN-β and the subsequent ISGs.

Since MAVS is mandatory to the RIG-I mediated response, HCV has evolved its NS3/4A protease to cleave the MAVS protein[1].

In order to understand the role of MAVS in HCV replication, MAVS$^{-/-}$ MEFs expressing miR-122 were electroporated with RNA transcribed from JFH-1 or JFH-1ΔGDD. Cells were cultured on 12-well plates and cell lysates were harvested 4 hours post electroporation (Day 0) and everyday for the next 5 days.

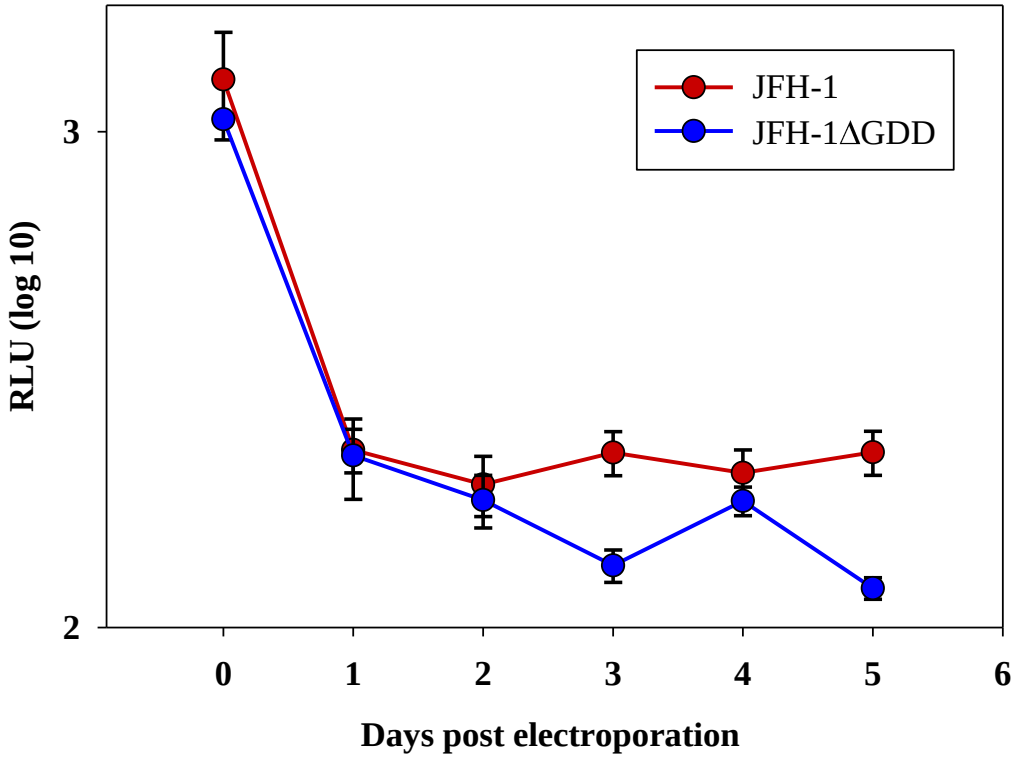

Figure 19 : Transient replication of JFH-1 and JFH-1ΔGDD in MAVS$^{-/-}$ MEFs

MAVS$^{-/-}$ MEFs expressing miR-122 were electroporated with JFH-1 or JFH-1ΔGDD RNA. Luciferase values were measured at 4h post electroporation (Day 0) as a read out for transfection efficiency and everyday for the next 5 days.

As shown above, no significant increase in luciferase expression derived from JFH-I RNA was observed in these cells in comparison to the polymerase mutant. This indicates that although MAVS protein in crucial in mediating a RIG-I dependent response, it is not the only pathway operating against HCV replication. The TLR-3 mediated TRIF pathway that activates cytosolic IRF-3 could be responsible in mediating an antiviral cellular state.

2.2 Chapter 2: Interferon independent pathways limiting HCV replication.

2.2.1 HCV replication induces interferon secretion in cells

The interferons are critical mediators of an antiviral response. The type I IFNs are the most frequently induced cytokines during an infection. These pleiotropic cytokines act in autocrine and paracrine ways to activate host cell antiviral responses and alert the surrounding cells of a viral invasion. The HCV RNA has specific structures coded by its genome that act as potent interferon inducers[117]. The poly (U/UC) tract at the 3'terminus and the 5'ppp are reported to be the major pathogen signatures recognized by RIG-I and the double stranded intermediates formed as a result of replication are recognized by TLR3. Within a cell, these molecular signatures are recognized by pattern recognition receptors that induce an interferon response, thereby establishing an antiviral state[63,117-120].

However, the evolutionarily evolved HCV has developed mechanisms to successfully evade these immune cascades. The HCV NS3/4A protein cleaves adaptors TRIF, MAVS and also successfully counteracts the STAT1 signalling mechanism. Therefore, upon HCV infection, interferon induction is severely compromised especially in cell culture and is difficult to detect. It was therefore important to analyze if fibroblasts transfected with the HCV replicon could be recognized by the PRRs and induce an interferon response. Additionally, since type I and type III interferons are capable of limiting HCV, the type of interferon produced by the fibroblasts was determined.

2.2.2 Replication of sub-genomic HCV replicon induces IFN secretion

HCV is reported to induce IFN in patients. Also, it is known that HCV is susceptible to IFN response. Therefore, it was important to determine if IFN was induced by HCV and if the mouse fibroblasts secreted IFN upon transfection. To this end, bioactive Interferon in the supernatant was quantified by luciferase expression in intestinal epithelial cell lines expressing luciferase under the control of the IFN-induced Mx2 promoter[121]. The IECs were incubated with supernatant harvested from MEFs electroporated with JFH-1 and the JFH-1ΔGDD strains. Reporter cell lines were incubated for 24 hours with supernatant

collected at several time points (Day 0, D1 and D2) post electroporation, and luciferase expression was measured.

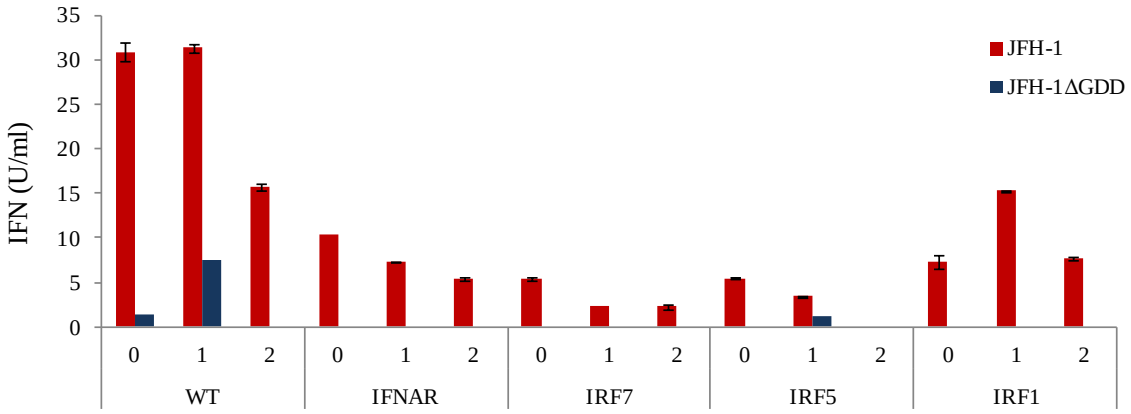

Figure 20 : Detection of Interferon secretion in HCV replicon transfected MEFs

Intestinal epithelial cells derived from an IFN reporter mouse carrying an Mx2 promoter-controlled luciferase construct [121] were stimulated with supernatants of the cells. Epithelial cells were incubated for 24 hours with supernatant from MEFs (WT, IFNAR$^{-/-}$, IRF-7$^{-/-}$, IRF-1$^{-/-}$ and IRF-5$^{-/-}$) electroporated with JFH-1 and the JFH-1ΔGDD for 4 hours (Day 0), day1 and day 2. The luciferase values were measured and quantified in U/ml.

As shown in **Figure 20**, upon transfection, the JFH-1 replicon induced IFN levels in all electroporated MEFs. The highest levels of IFN secreted was measured in WT MEFs (30U/ml) while IFNAR$^{-/-}$, IRF-7$^{-/-}$, IRF-1$^{-/-}$ and IRF-5$^{-/-}$ showed less but detectable levels of IFN. However, the JFH-1ΔGDD induced detectable IFN levels in WT and IRF-1$^{-/-}$ MEFs. This could be explained as a result of cellular recognition of molecular patterns of transfected RNA.

Taken together, these data indicate that the JFH-1 and the JFH-1ΔGDD encode pathogen signatures that are recognized by the cellular interferon machinery. However, the JFH-1 strain, capable of replication induces higher levels of interferon in comparison to the NS5B-deficient strain. This can be explained by the double stranded intermediates that the positive sense single stranded HCV RNA forms during the process of replication. These intermediates act as inducers of the TLR3 signalling pathway[122].

Taken together, these data indicate that both JFH-1 and JFH-ΔGDD HCV RNA can induce a detectable interferon response. This suggests that the HCV replicon encodes in itself molecular signatures that can be detected by the host immune system. Additionally, replication competent HCV induced IFN secretion much higher than the polymerase

deficient strain suggesting that although HCV replicon specific structures could induce an interferon response, replicating HCV replicons induced much higher levels of interferon.

2.2.3 Mouse fibroblasts secrete type I interferon

Both, type I IFN and type III IFN are shown to induce antiviral responses restricting HCV replication[101,102]. Bioactive Interferon in the supernatant was quantified by luciferase expression in intestinal epithelial cell lines expressing luciferase under the control of the IFN-induced Mx2 promoter[121]. Intestinal epithelial cells are sensitive to Type I and III IFN as intestinal epithelia posses both the type I and the type III IFN receptors. Supernatant was harvested from electroporated MEFs that were cultured in the presence or absence of neutralizing antibodies against type I interferon at 4 hours (Day 0), day 1 and day 2 post electroporation. Intestinal epithelial cells were incubated with the supernatant for 24 hours and the luciferase values were measured.

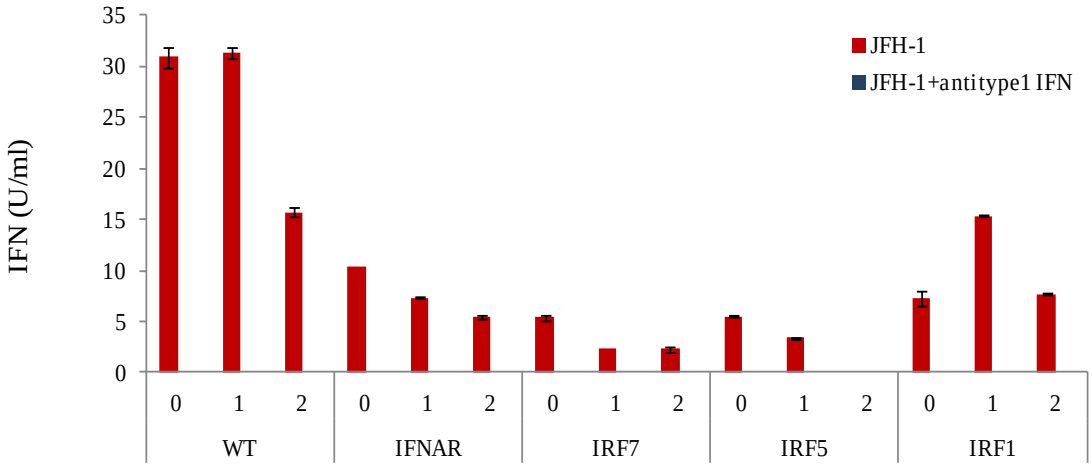

Figure 21 : MEFs secrete Type I Interferon

For the IFN bioassays, supernatants of WT, IFNAR$^{-/-}$, IRF-7$^{-/-}$, IRF-5$^{-/-}$ and IRF-1$^{-/-}$ cells electroporated with RNA from JFH1 were harvested 4h (0) or 1 and 2 days post electroporation. Reporter intestinal epithelial cells derived from an IFN reporter mouse carrying an Mx2 promoter-controlled luciferase construct [121] were stimulated with supernatants of the cells. Luciferase activity was determined after 24h. To determine the IFN species, cells were incubated with 2 µg/ml of IFNα (4EA1) and IFN-β (7FD3) antibodies neutralizing 500U of IFN. IFN concentrations were obtained by administering serial dilutions of IFN-β. The luciferase values were measured and quantified in U/ml.

The experiment demonstrates that replicating JFH-1 is capable of inducing detectable levels of interferon in all transfected MEFs as measured by the Mx2 luciferase reporter assay. Additionally, when type I IFN was neutralized, luciferase expression was completely

abolished suggesting that Mx2 induction was solely dependent on the type I IFN response and not the type III IFNs. This indicates that mouse fibroblasts secrete type I IFN following HCV replication.

2.2.4 Blocking the paracrine IFN response does not rescue HCV replication in WT MEFs

Interferon secreted by a cell upon viral encounter is taken up by specific IFN receptors to aid amplification of this response. In addition to the infected cell, the paracrine mode of IFN signalling ensures the establishment of an antiviral state in neighbouring cells. Since the secretion of interferon by the electroporated cells has been established, their plausible influence on HCV replication was analyzed. WT MEFs electroporated with polymerase competent or deficient JFH-1 RNA were cultured on 12-well plates in the presence of absence of neutralizing antibodies (2µg/ml) against Type I IFN. Cells were harvested at 4 hours post electroporation (Day 0) to determine transfection efficiency and everyday for the next 5 days.

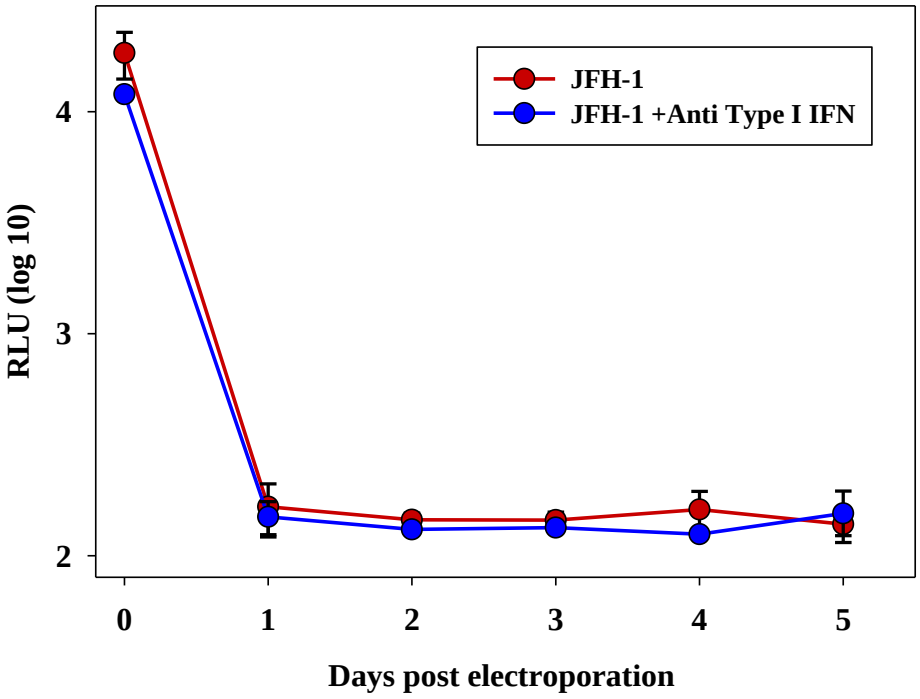

Figure 22 : Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in WT MEFs

WT MEFs were electroporated with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

As shown in **Figure 22**, there was no detectable expression of luciferase expression of JFH-1 replicon. Expression levels were comparable to the JFH-1ΔGDD replicon**.** Treatment of the cells with neutralizing antibodies against type I IFNs had no influence on the replication of the HCV subgenomic replicon. This suggests that neutralization of secreted IFN could not rescue HCV replication in WT mouse fibroblasts indicating that although HCV restriction depends on type I IFN receptor **Figure 9**, the presence of more restriction factors than type I interferon.

2.2.5 Neutralizing type I IFN response does not enhance HCV replication in IFNAR$^{-/-}$ MEFs

Although MEFs with a lesion in the IFN receptor are not responsive to IFNs, they are capable of producing IFN in response to a pathogen attack. This response is however limited due to the defect in the continued amplification of the initial response. Therefore, cells and animals deficient in the IFN receptor have been shown to be extremely susceptible to viral infections.

To determine if replication could be enhanced in the absence of a positive IFN amplification loop, cells were electroporated with RNA transcribed from JFH-1 replicon and cultured in the presence or absence of neutralizing antibodies against IFN-α and IFN-β. The cells were harvested at 4 hours post transfection (Day 0) and everyday for the next 5 days.

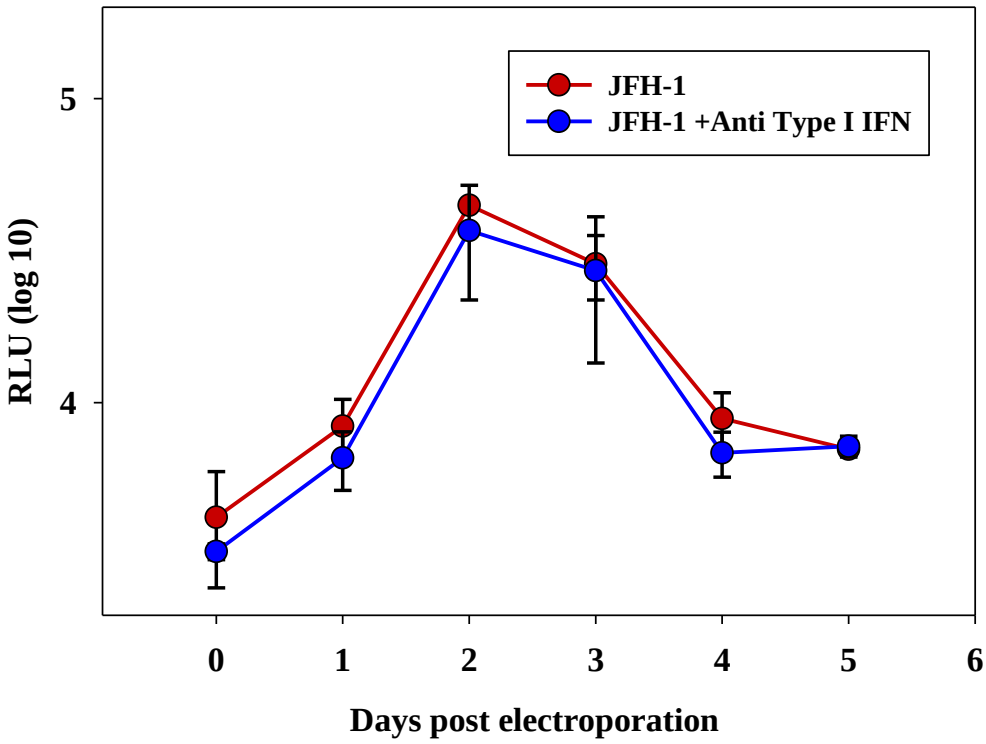

Figure 23 : Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in IFNAR1$^{-/-}$ MEFs

IFNAR1$^{-/-}$ MEFs were electroporated with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

Although IFN secreted from these cells could be detected, depletion of IFN-α and IFN-β by neutralizing antibodies did not further increase HCV replication.

Taken together, this indicates that in addition to restriction factors operating at the level prior to IFN induction, the positive amplification of IFN via the IFN receptor is indispensible to HCV restriction in mouse fibroblasts and as expected, replication is not enhanced further by neutralizing secreted IFNs.

2.2.6 IRF-3 dependent HCV restriction

The role of another interferon regulatory factor, IRF-3 has been shown to be important in limiting HCV in mouse fibroblasts. IRF-3 has been shown to induce antiviral ISGs directly and is responsible for inducing early type I interferon. Although HCV was shown to replicate in the absence of IRF-3, it was tested if the additional deletion in the IFN amplification loop could enhance HCV replication. To this end, IRF-3$^{-/-}$ MEFs were

electroporated with RNA transcribed from JFH-1 replicon and cultured in the presence or absence of neutralizing antibodies against IFN-α and β. Cells were harvested at 4 hours post electroporation (Day 0) to determine transfection efficiency and everyday for the next 5 days.

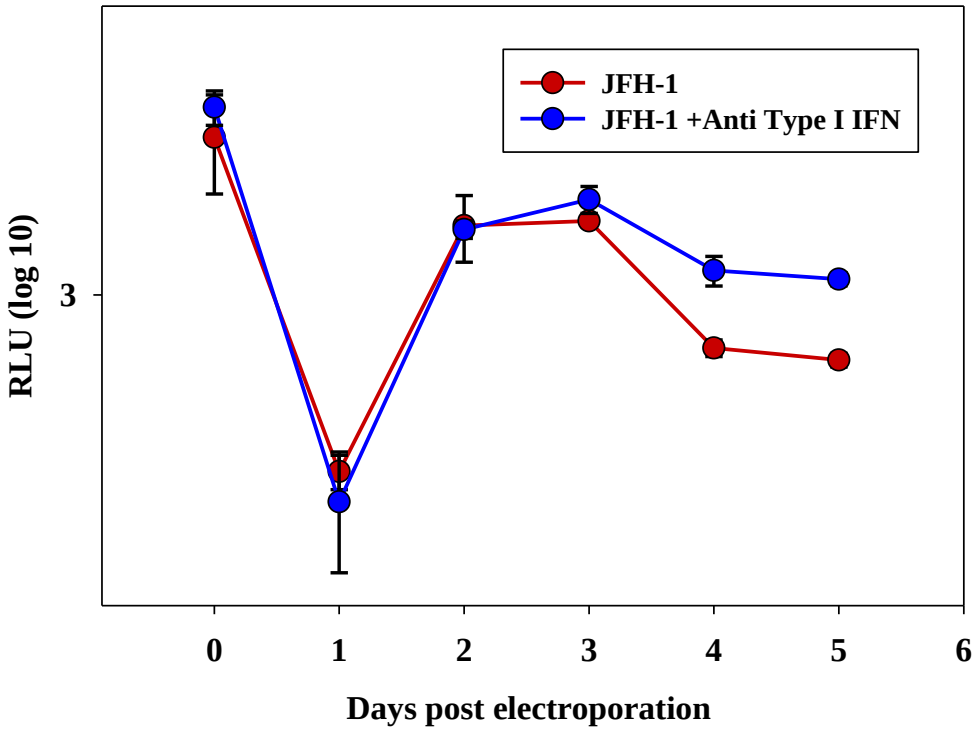

Figure 24 : Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in IRF-3$^{-/-}$ MEFs

IRF-3$^{-/-}$ MEFs were electroporated with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

As depicted above, in the IRF-3$^{-/-}$ MEFs, the added lesion in the IFN amplification loop did not further enhance HCV replication in comparison to when only IRF-3 was deleted. This indicates that IRF-3 alone is sufficient to restrict HCV replication, may be because no IFN is induced in the absence of IRF-3.

2.2.7 STAT1 induced restriction of HCV replication

STAT1 is an important transcription factor that induces downstream interferon stimulated genes in response type I, II and type III interferon. The STAT1 proteins are crucial in antiviral defence and hence it is not surprising that viruses have evolved strategies to evade the STAT1 response. The core protein of HCV has been shown to associate with STAT1

and induce degradation in a proteasome-dependent manner. Additionally, the HCV NS5A has been observed to inhibit STAT1 phosphorylation, suppressing nuclear translocation and the ensuing immune response.

In order to validate the IFN-independent role of STAT1, mouse fibroblasts deficient in STAT1 were utilized to study HCV replication.

To this end, immortalized STAT1$^{-/-}$ MEFs were electroporated with RNA transcribed from JFH-1 replicon and cultured in the presence or absence of neutralizing antibodies against IFN-α and β. Cells were harvested at 4 hours post electroporation (Day 0) to determine transfection efficiency and everyday for the next 5 days.

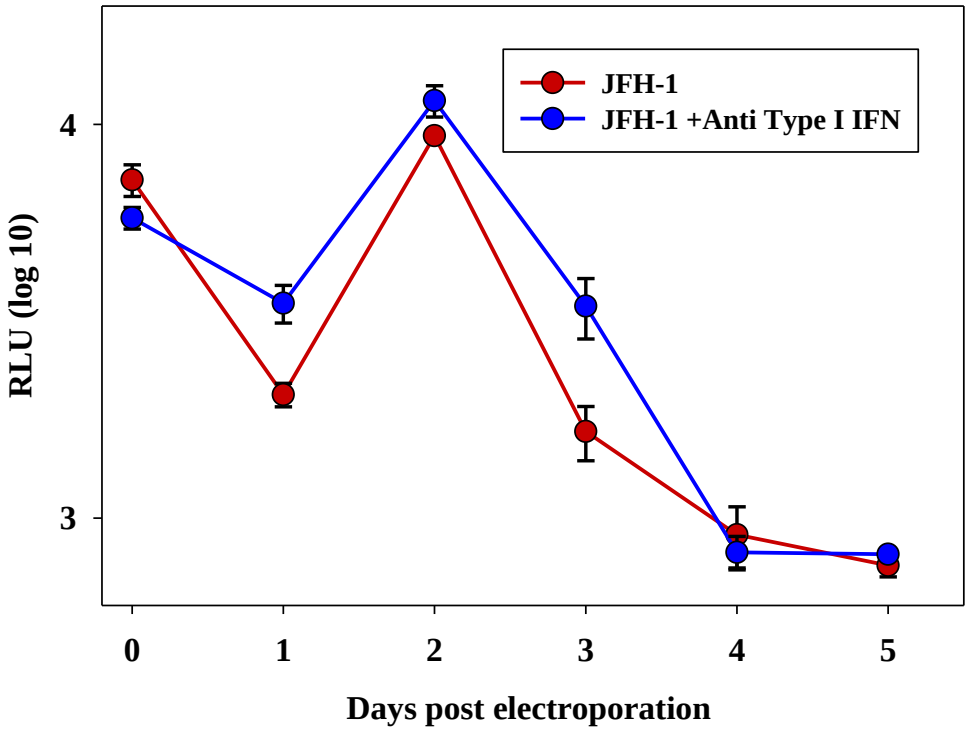

Figure 25 : Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in STAT1$^{-/-}$ MEFs

STAT1$^{-/-}$ MEFs were electroporated with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3)

As shown above, no additional luciferase expression was observed following neutralization of the type I interferon response.

STAT proteins complex with IRF-9 to form the ISGF3 complex that translocates to the nucleus and results in their binding to the consensus elements on promoters of interferon stimulated genes. This suggests that although the STAT1 is required for limiting HCV

replication by amplifying the IFN response, it is dependent on the positive amplification of the type I IFN and the induction of ISGs.

2.2.8 IFN independent pathways for limiting HCV replication

The interferon system has evolved to protect cells from a viral attack. Therefore, in the context of a viral infection, the IFN system is particularly indispensible. Although the regulators of the interferon system (the IRFs) are largely homologous and redundant in their activities and pivotal in protecting an infected cell, it is believed that the interferons evolved to protect uninfected neighbouring cells as well. This suggests that in addition to IFN driven antiviral responses, IRF mediated antiviral pathways may play a role in viral defence[5,123]. Since no rescue of HCV was observed in WT MEFs upon disabling the IFN response, we hypothesized the presence of an additional factor prior to the IFN amplification loop aiding in limiting HCV replication.

In order to test the impact of these IFN-independent antiviral pathways, MEFs, individually deficient in interferon regulatory factors were tested for their ability to support HCV replication in the combined absence of the IFN amplification loop.

IRF-5 has been studied in relation with autoimmune diseases and its role in antiviral immunity is emerging. When present in the cell, it is found in its latent form which gets activated upon infection and translocates to the nucleus. Although morphine [124] and methamphetamine [125] have shown to suppress the expression of p38 and IRF-5 respectively increasing HCV replication in hepatocytes, the role of IRF-5 in limiting HCV is still largely elusive.

In order to ascertain the role of IRF-5 in the absence of a functional interferon response, MEFs deficient in IRF-5 were utilized to analyze the replication of HCV.

To this end, IRF-$5^{-/-}$ MEFs were electroporated with RNA transcribed from JFH-1 replicon and cultured in the presence or absence of neutralizing antibodies against IFN-α and β. Cells were harvested at 4 hours post electroporation (Day 0) to determine transfection efficiency and everyday for the next 5 days.

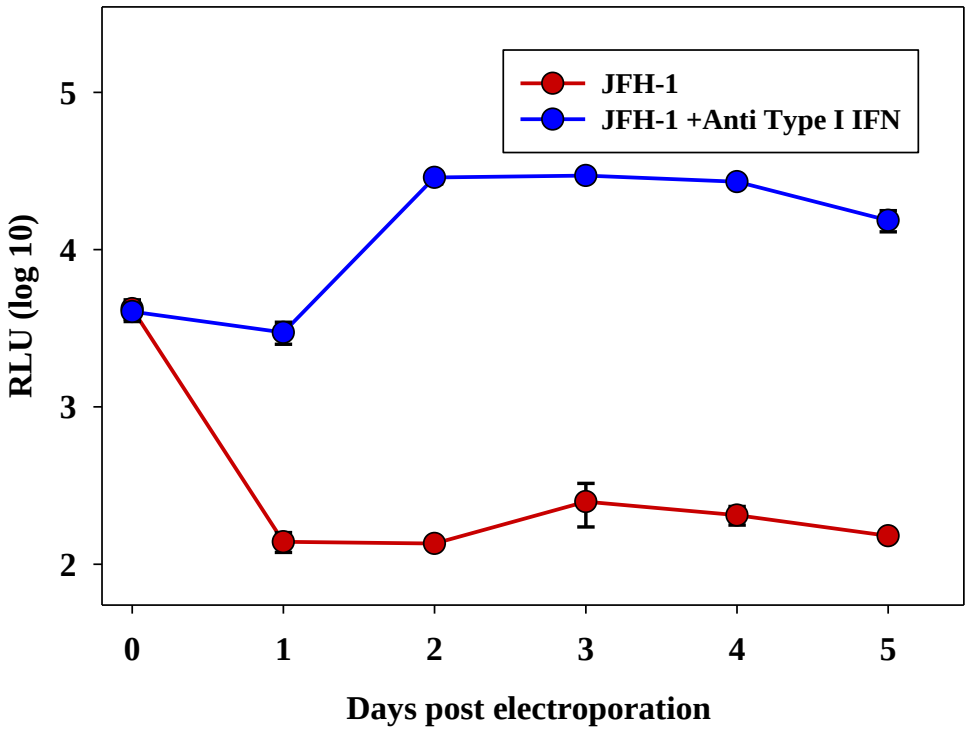

Figure 26 : Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in IRF-5$^{-/-}$ MEFs

IRF-5$^{-/-}$ MEFs were electroporated with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

The data shown above indicate that the absence of IRF-5 (red line) alone is not sufficient to sustain HCV replication. However, in the added deletion of the IFN response, HCV replication can be observed.

The significant increase in HCV replication compared to when only IRF-5 was deleted suggests the presence of additional antiviral mechanisms dependent on IRF-5 but independent of type I interferons.

IRF-7 is involved in the amplification of interferon responses and is also induced by interferon. Although HCV has been shown to functionally inhibit IRF-7 by effectively blocking nuclear translocation [126] in hepatocytes, it was observed to be dispensable for limiting HCV replication in mouse fibroblasts. In order to ascertain the IFN- independent role of IRF-7 in limiting HCV replication, mouse fibroblasts isolated from IRF-7$^{-/-}$ mice were studied for HCV replication.

To this end, IRF-7$^{-/-}$ MEFs were electroporated with RNA transcribed from JFH-1 replicon and cultured in the presence or absence of neutralizing antibodies against IFN-α and β.

Cells were harvested at 4 hours post electroporation (Day 0) to determine transfection efficiency and everyday for the next 5 days.

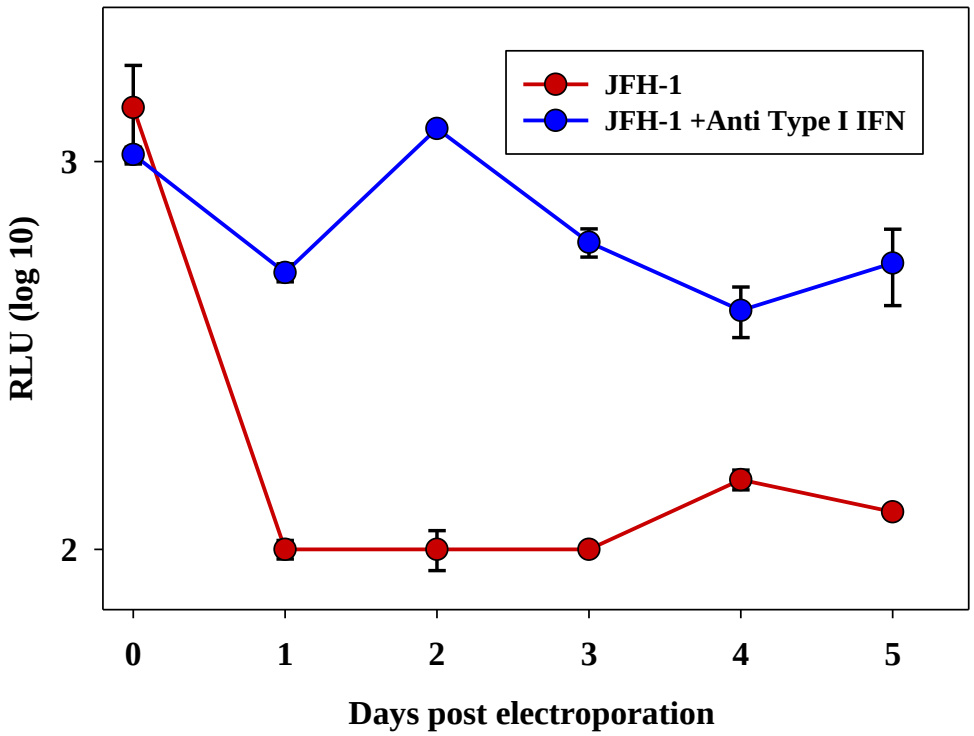

Figure 27 : Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in IRF-7$^{-/-}$ MEFs.

IRF-7$^{-/-}$ MEFs were electroporated with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3)

As shown above, the JFH-1 replicon could not replicate in the absence of IRF-7. However, in the combined absence of IRF-7 and the IFN signalling pathway a rescue in HCV replication could be observed. This suggests a plausible function of IRF-7 in limiting HCV replication.

Similarly, the role of IRF-1 in HCV viral clearance and response to treatment has been elucidated in patient cohorts[115]. Therefore, in order to elucidate the IFN-independent antiviral function of IRF-1, HCV replication was studied in IRF-1$^{-/-}$ MEFs.

To this end, IRF-1$^{-/-}$ MEFs were electroporated with RNA transcribed from JFH-1. The cells were cultured on 12-well plates in the presence or absence of neutralizing antibodies against IFN-α and β. Cells were harvested at 4 hours post electroporation (Day 0) to determine transfection efficiency and everyday for the next 5 days.

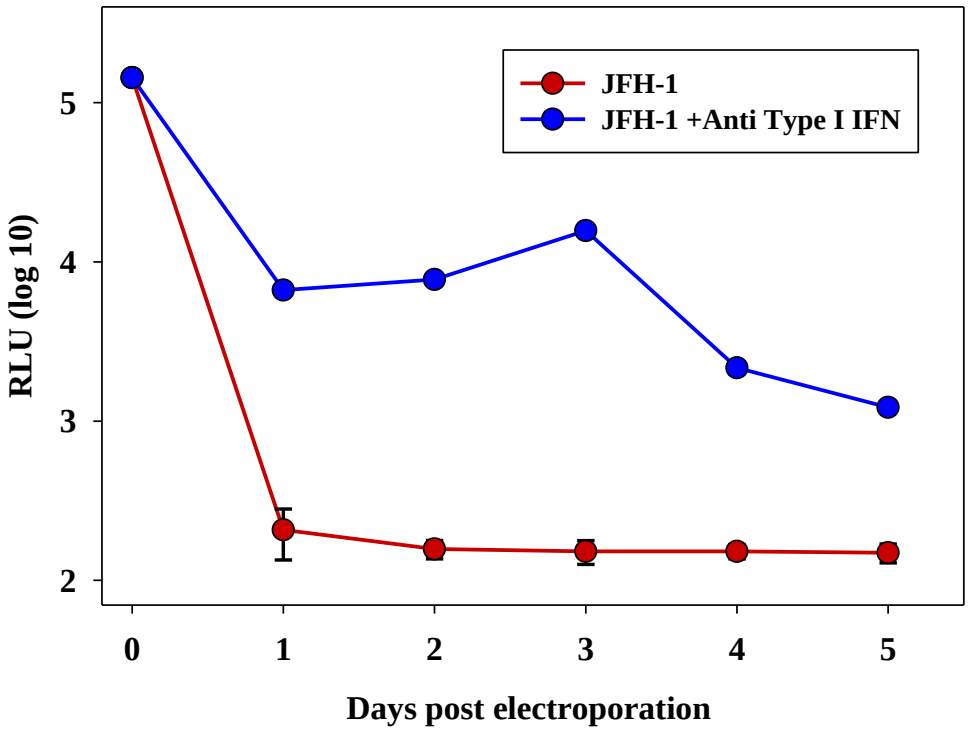

Figure 28 : Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in IRF-1$^{-/-}$ MEFs

IRF-1$^{-/-}$ MEFs were electroporated with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

As shown above, the data indicate an increased replication of the JFH-1 replicon in the absence of IRF-1 and IFN amplification when compared to only in the absence of IRF-1

In comparison to the WT MEFs with a fully able innate immune system, where no replication of HCV was observed upon blocking the paracrine response, HCV was seen to replicate in the additional absence of IRF-1. This suggests the potential role of IRF-1 in limiting HCV replication in the absence of a type I IFN response.

Protein kinase R (PKR) has been reported to be pivotal in antiviral defence. Following binding to dsRNA, PKR dimerizes and undergoes autophosphorylation. Thus activated, PKR phosphorylates eIF2A suppressing the translation machinery. In order to circumvent the lack of the host translational machinery, HCV has developed a functional IRES element. The IRES element aids in ribosome-mediated translation of the viral proteins.

In order to determine the role of PKR in an IFN-independent setting, $PKR^{-/-}$ MEFs were assayed for HCV replication.

To this end, $PKR^{-/-}$ MEFs were electroporated with RNA transcribed from JFH-1 replicon and cultured in the presence or absence of neutralizing antibodies against IFN-α and β. Cells were harvested at 4 hours post electroporation (Day 0) to determine transfection efficiency and everyday for the next 5 days.

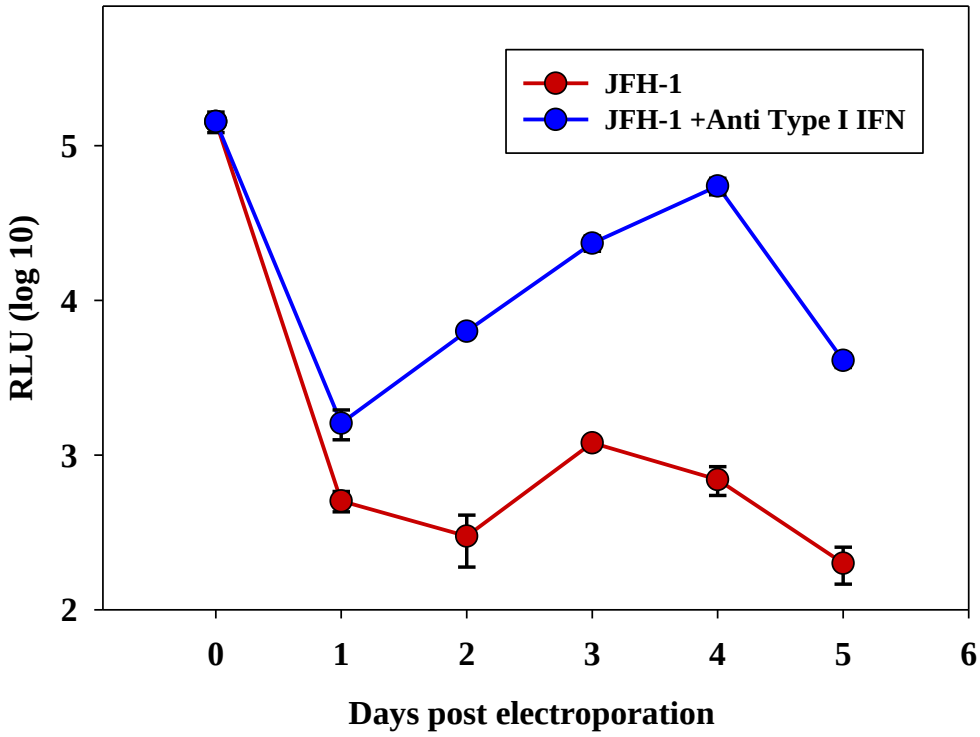

Figure 29 : Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in $PKR^{-/-}$ MEFs

$PKR^{-/-}$ MEFs were electroporated with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

As depicted above, although slight luciferase expression could be determined in the absence of PKR, the combined absence of PKR and the type I IFN amplification loop permitted enhanced replication. This suggests that PKR functions in inhibiting HCV replication in a manner independent of the type I IFN response. Because PKR expression is dependent of type I IFN, depletion of constitutive low levels of type I IFN could reduce PKR expression and therefore PKR dependent restriction of HCV replication.

2.3 Chapter 3: Generation of an inducible HCV cell line

The understanding of the pathogenesis and persistence of the Hepatitis-C virus has been severely hindered due to the lack of suitable cell culture systems. Most available cell systems are based on human hepatoma cell lines such as Huh7 and its derivatives. The interferon and the ensuing interferon stimulated genes (ISGs) are pivotal in limiting HCV. Data generated from infection studies on chimpanzees reveal differential outcomes based on the time of induction of ISGs following an infection. It has been observed that immediate to high induction of ISGs upon infection results in acute infection which is later cleared, whereas a decreased or delayed ISG response to infection is associated with chronicity in chimpanzees experimentally infected with HCV[100].

Transient transfection of cells in culture is limited by the number of cells 'hit', ie: not all cells are positively transfected leading to an 'asynchronic' response. In order to circumvent this varied response, a plasmid coding for an HCV subgenomic replicon JFH-1 was cloned downstream of an inducible Tet promoter. An inducible promoter allows for the expression of the cassette under specific permissive conditions.

The advantage of a Tet dependent construct coding for the JFH-1 replicon is that it can be induced by supplementing cell culture media with a Tetracycline derivative (Doxycycline). Co-transfection of a construct containing a KRAB repressor allows for strict expression of the gene of interest only under permissive conditions thereby avoiding possible leaky expression. The expression of the cassette is based on the 'Tet-off' system wherein the gene of interest is maintained at a 'switched-off' mode in the presence of Doxycycline.

Apart from the advantage of inducibility in gene expression, clonal expansion of cells stably expressing the plasmid results in a pool of cells responding in a synchronous manner. Importantly, this system permits the identification of interferon responses immediately after encountering the viral genome. This is important as the first set of ISGs induced by the activated IRF-3 is relatively less and difficult to detect especially if only a fraction of the cell population is transfected. A synchronic cellular response against the viral genome permits detection of early immune responses.

2.3.1 Establishment of Huh7.5 cells expressing a stable Tet inducible HCV subgenoimc replicon

Huh7.5 cells are human hepatoma cells derived from the Huh7 cell line. Hepatoma cells exhibit downregulated TLR3 expression[2]. In addition to deficient TLR3, these cells have an additional lesion in the RIG-I making Huh7.5[118] cells permissive to HCV replication.

The aim was to generate Huh7.5 cells stably expressing a plasmid coding for a Tet inducible HCV replicon to establish a tool which allows the identification of early cellular ISG response upon HCV replication.

Huh7.5 cells stably expressing inducible JFH-1 and the NS5B-deficient JFH-1ΔGDD subgenoimc replicons were generated. To this end, the Huh7.5 cells were transfected with a plasmid containing the JFH-1 or the JFH-1ΔGDD subgenoimc replicon cloned downstream of a Tet promoter (**Figure 30** A), and co-transfected with a lentiviral vector containing the transactivator including the rKRAB sequence. The KRAB repressor under the constitutive PGK promoter ensures tight expression whereas the presence of a mutated nerve growth factor acts as a surface marker enabling detection of transfected cells by immunoflourescence or microscopy. The PGK driven KRAB repressor is connected to the transactivator through an EMCV-IRES sequence.

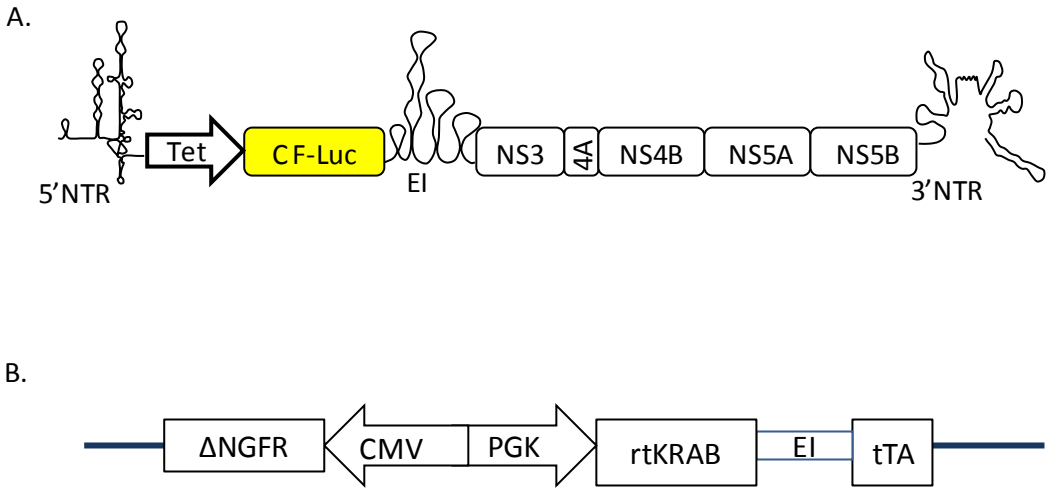

Figure 30: Schematic representation of A. The tet inducible HCV plasmid and B. the lentiviral plasmid coding for the transactivator.

A. The Tet promoter is introduced in front of the JFH-1 subgenoimc replicon. **B**. The third generation self-inactivating lentiviral plasmid expressing the KRAB repressor connected to a transactivator by an EMCV-IRES (EI) transcriptionally driven by the PGK promoter. Additionally, a mutated nerve growth factor receptor is driven by the CMV promoter.

2.3.2 The Tet dependent plasmids show inducible expression

In order to test the inducibility of the plasmid containing the tetracycline-inducible HCV subgenomic replicon, Huh7.5 cells were transiently transfected with the inducible replicons (JFH-1-prom3 or JFH-1ΔGDD-prom3) and the plasmid containing the transactivator (Lenti tTA). Transfection efficiency was normalized by co-transfection of a Renilla Luciferase plasmid.

Upon transfection, the cells were maintained in the absence of Doxycycline for three days in order to aid Tet-dependent transcription. Following this, the cells treated with or without Doxycycline (2µg/ml) and the luciferase values were recorded daily for a period of 5 days.

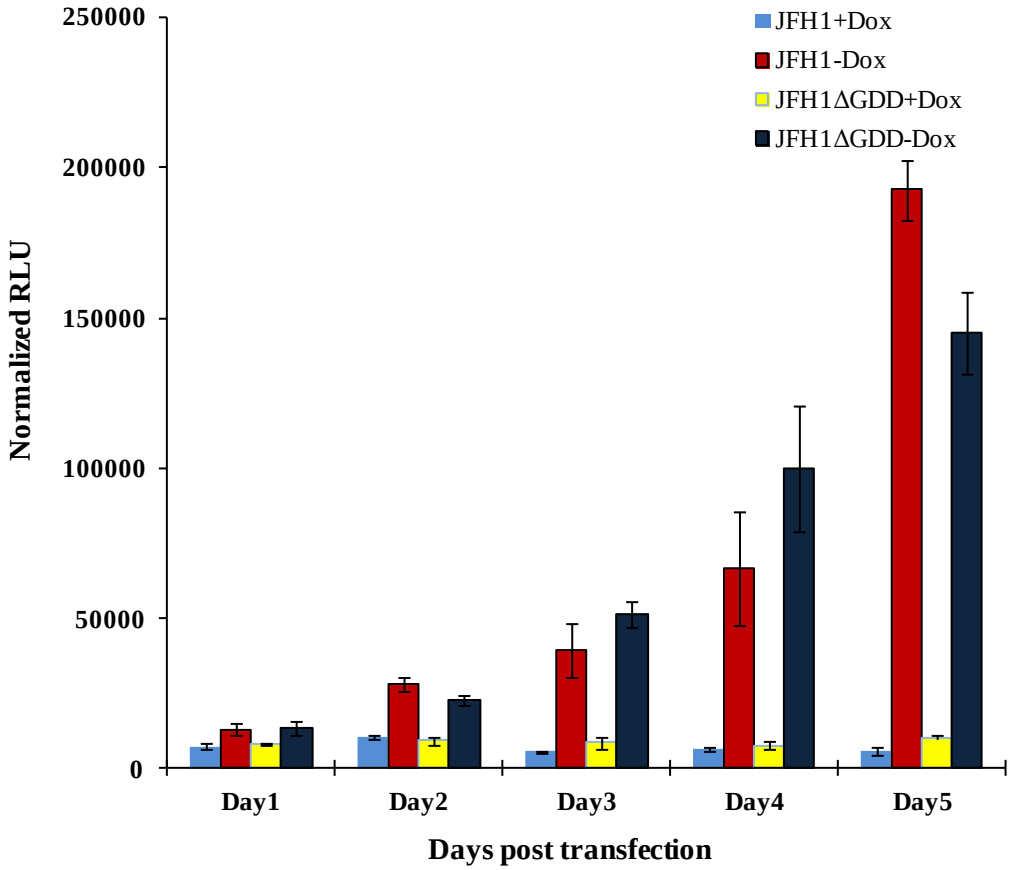

Figure 31: **Inducible expression of the HCV replicons**.

The Tet inducible plasmids JFH-1 and IFH-1ΔGDD were transfected in the presence of a lentiviral plasmid coding for the transactivator along with the reporter plasmid pMdicR-luc. Upon transfection, the cells were maintained in the absence of Doxycycline for three days. Following this, the cells treated with or without Doxycycline (2µg/ml) and the luciferase values were recorded everyday for a period of 5 days. Luciferase values were measured and normalized to the Renilla luciferase values.

Luciferase values were measured in cells incubated in the presence or absence of Dox each day for up to 5 days. The normalized values revealed increasing levels of luciferase expression in the absence of Doxycycline with time. This is a result of continued transcription in the absence of Doxycycline (red and dark blue bars). Additionally, the slight increase in luciferase expression levels observed in the JFH-1 replicon (red bars) in comparison to the JFH-1ΔGDD (dark blue bars) in the absence of Doxycycline could be a result of NS5B enabled replication of the JFH-1 strain.

In contrast, when cells are cultured in the presence of Dox, the Tet-induced transcription is switched off and no luciferase expression is observed in the JFH-1 (light blue bars) and the JFH-1ΔGDD constructs (yellow bars).

Taken together, these data suggest that the absence of doxycycline induces Pol II dependent transcription in both the inducible JFH-1 as well as the JFH-1ΔGDD constructs. Additionally, the slight increase in luciferase expression observed in the JFH-1 and the JFH-1ΔGDD constructs could be due to NS5B polymerase dependent replication.

Since **Figure 31** establishes that transcription of these constructs could be regulated, the increase in luciferase expression of the JFH-1 in comparison to JFH-1ΔGDD was attributed to plausible replication of the JFH-1 replicon. To this end, the plasmids were tested for their capacity to replicate following Dox induced transcription.

2.3.3 The plasmids exhibit the potential to replicate

Transient induction assays of JFH-1-prom3 showed a slight increase in luciferase expression in comparison to the JFH-1ΔGDD-prom3 construct. This trend was hypothesized to be due to the added ability of the JFH-1-prom3 to replicate aided by the viral polymerase NS5B. The three amino acid mutation in the JFH-1ΔGDD disrupts viral polymerase inhibiting the construct from replicating.

In order to verify if the polymerase competent strain (JFH-1-prom3) could initiate and maintain replication after Tet induction, a replication assay in the presence of NS5B polymerase inhibitor 2-CMA was performed. To this end, Huh7.5 cells transiently transfected with JFH-1-prom3 (POL+) or JFH-1ΔGDD-prom3 was co-transfected with lenti tTA and a transfection control vector expressing Renilla luciferase. Upon transfection, the cells were maintained in the absence of Doxycycline for three days in order to aid Tet dependent transcription. Following this, the cells were cultured in media supplemented with

Doxycycline in the presence or absence of the NS5B polymerase inhibitor 2-CMA. Luciferase values were measured after 29 hours and 39 hours of incubation.

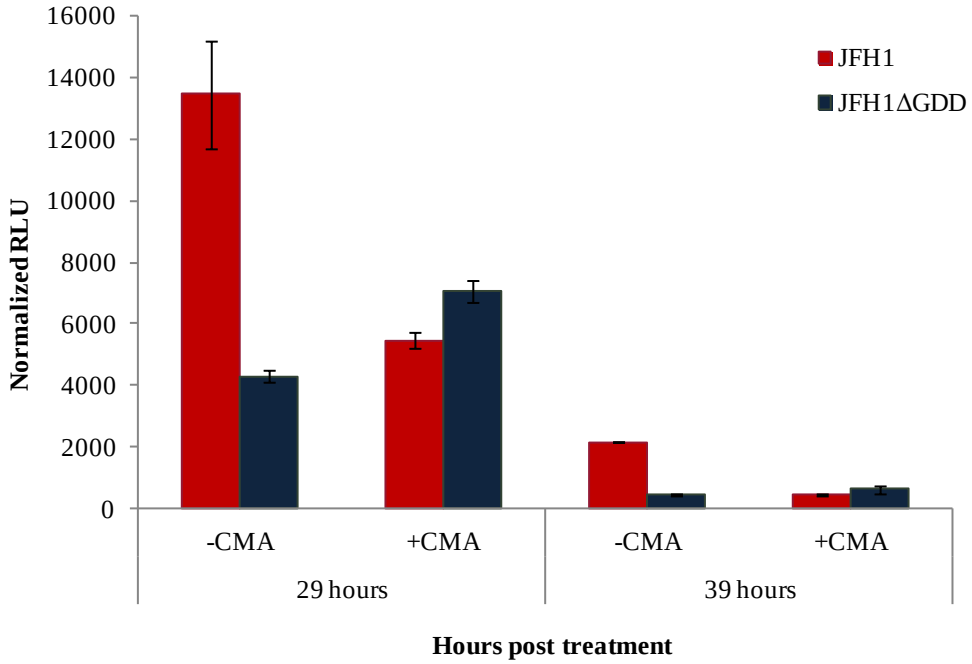

Figure 32: Testing the replication potential of the tet inducible plasmids.

The Tet inducible plasmids JFH-1 and IFH-1ΔGDD were transfected in the presence of a lentiviral plasmid coding for the transactivator along with the reporter plasmid Mdic R-luc. Upon transfection, the cells were maintained in the absence of Doxycycline for three days. Following this, the cells treated with or without polymerase inhibitor (2-CMA). Luciferase values were measured and normalized to the Renilla luciferase values.

These data showed an increased level of luciferase expression in cells transfected with the JFH1 plasmid in the absence of 2-CMA in comparison to the JFH-1ΔGDD. However, in the presence of 2-CMA, a polymerase inhibitor, the luciferase expression of the cells transfected with JFH1 construct was comparable to the cells transfected with JFH-1ΔGDD. This suggests that difference in luciferase levels observed between the two constructs in the absence of 2-CMA is due to the replication potential of the JFH1 construct.

This indicates that the inducible JFH1 construct exhibits the potential to replicate using the viral polymerase NS5B whereas the JFH-1ΔGDD-prom3 is unable to do the same.

This system has allowed for the generation of novel inducible HCV cell lines using which interferon stimulated genes induced at very early time points, such as immediately after the host recognizes the virus can be analyzed.

2.4 Chapter 4: HCV replication analysis in primary mouse hepatocytes

Hepatitis-C virus is a largely hepatotropic virus. Although HCV research is carried out extensively on hepatoma cell lines, primary hepatocytes prove to be a good host system to study HCV infection or replication. Primary human hepatocytes have been widely used for analysis of pathology and toxicology. However, extensive research is limited due to limited sample availability, comparably low efficiency and reproducibility. Also, the role of individual proteins of the interferon response can only be carried out upon knocking down these effectors.

Although primary murine hepatocytes are not naturally susceptible to HCV infection, they can be used to characterize the replication of tranfected HCV RNA. The use of primary murine hepatocytes in studying HCV replication has several advantages. Firstly, owing to the availability of mice strains deficient in specific essential proteins of the interferon system, the importance of these factors in HCV replication can be studied. Secondly, unlike hepatoma cell lines with mutated pathogen recognition receptors and the ensuing interferon response, primary hepatocytes have a fully competent interferon system unless otherwise altered. Since hepatocytes are highly differentiated cells, they must be cultured under stringent conditions to maintain their regular structure and physiology. A significant disadvantage of primary heaptocyte culture is its high propensity to de-differentiate into fibroblast-like cells[127].

2.4.1 HCV replication in WT hepatocytes

The aim here was to investigate HCV replication in hepatic cells and to elucidate the role of the IFN system as restriction factors for HCV replication.

Primary murine hepatocytes were isolated from WT mice and transfected with *in vitro* transcribed RNA from JFH-1 subgenoimc replicon as well as the polymerase mutant JFH-1ΔGDD. Luciferase levels as a measurement of HCV replication were determined 5 hours post transfection and every day for the following 3 days.

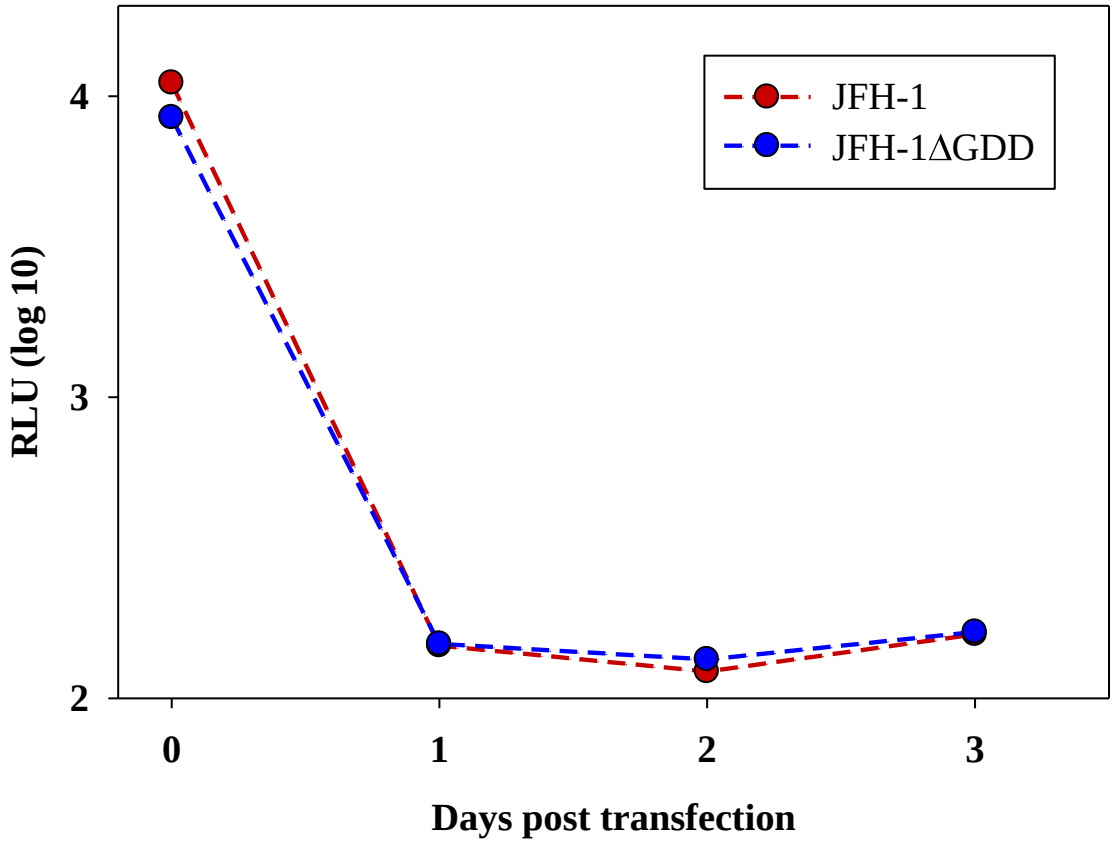

Figure 33: HCV replication in WT primary hepatocytes

Primary hepatocytes isolated from WT mice were transfected with JFH-1 (red line) or the polymerase deficient JFH-1ΔGDD (blue line) RNA. Luciferase values were measured at 5h post transfection (Day 0) as a read out for transfection efficiency and everyday for the next 3 days.

As shown in **Figure 33**, luciferase expression dropped after 5 hours and did not increase in the days following lipofection. This indicates that HCV was unable to replicate effectively in WT hepatocytes. Since data presented in MEFs (**Figure 9**) discusses the inhibitory role of type I interferon, we tested if type I interferon was inhibiting HCV replication. To this end, we tested viral replication in hepatocytes derived from WT mice in the presence of neutralizing type I IFN antibodies.

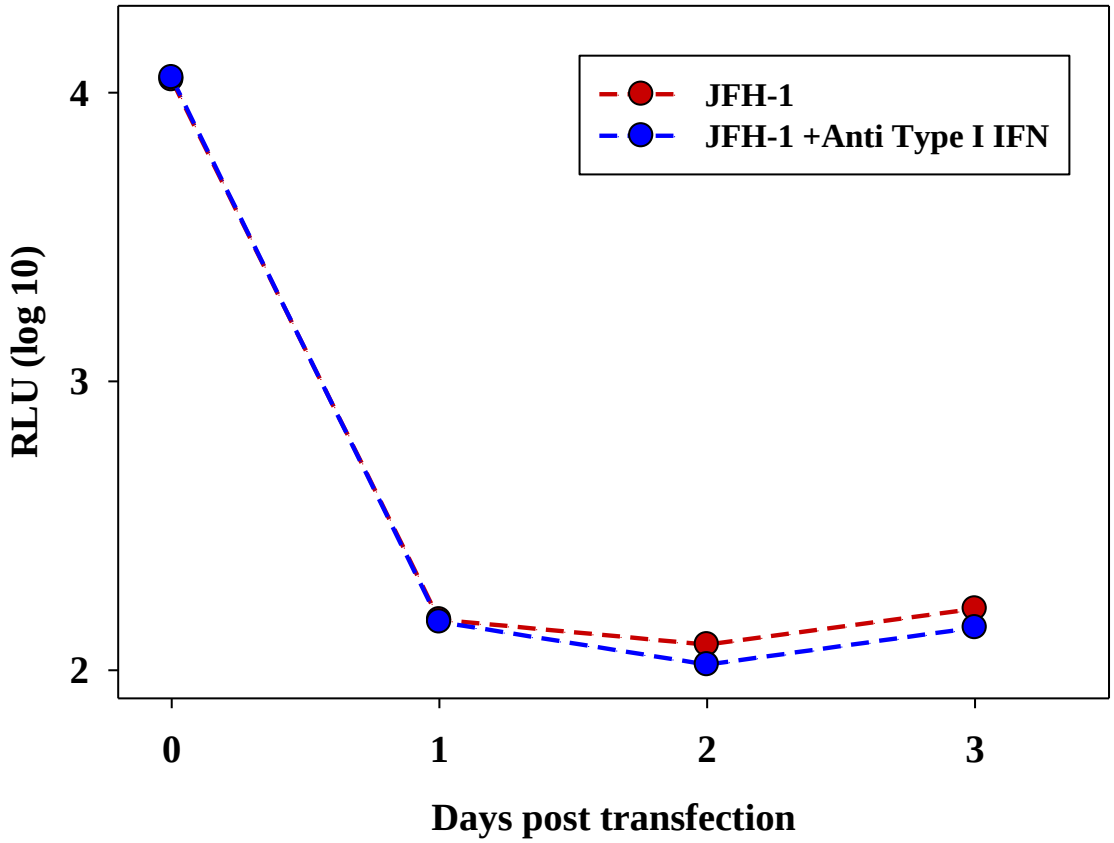

Figure 34: Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in WT hepatocytes

WT hepatocytes were transfected with JFH-1 RNA. The electroporated cells were cultured on 12-well plates without (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

Despite the blocking of the type I interferon response, the same pattern was observed in cells treated with antibodies against type I interferon. This indicates that the WT hepatocytes could not maintain replication of tranfected HCV replicon RNA. These data reflect the data from WT MEFs where depletion of the type I interferon response could not rescue HCV replication.

2.4.2 HCV replication in IFNAR$^{-/-}$ hepatocytes

In order to verify if type I interferon was the major restriction factor for HCV replication in mouse hepatocytes as was observed to be in fibroblasts, IFNAR deficient hepatocytes were tranfected with HCV RNA and replication potential was analysed. To this end, primary hepatocytes with a defective type I IFN receptor were transfected with RNA from the JFH-1 subgenoimc replicon. Luciferase levels were determined 5 hours post transfection and for the following 3 days.

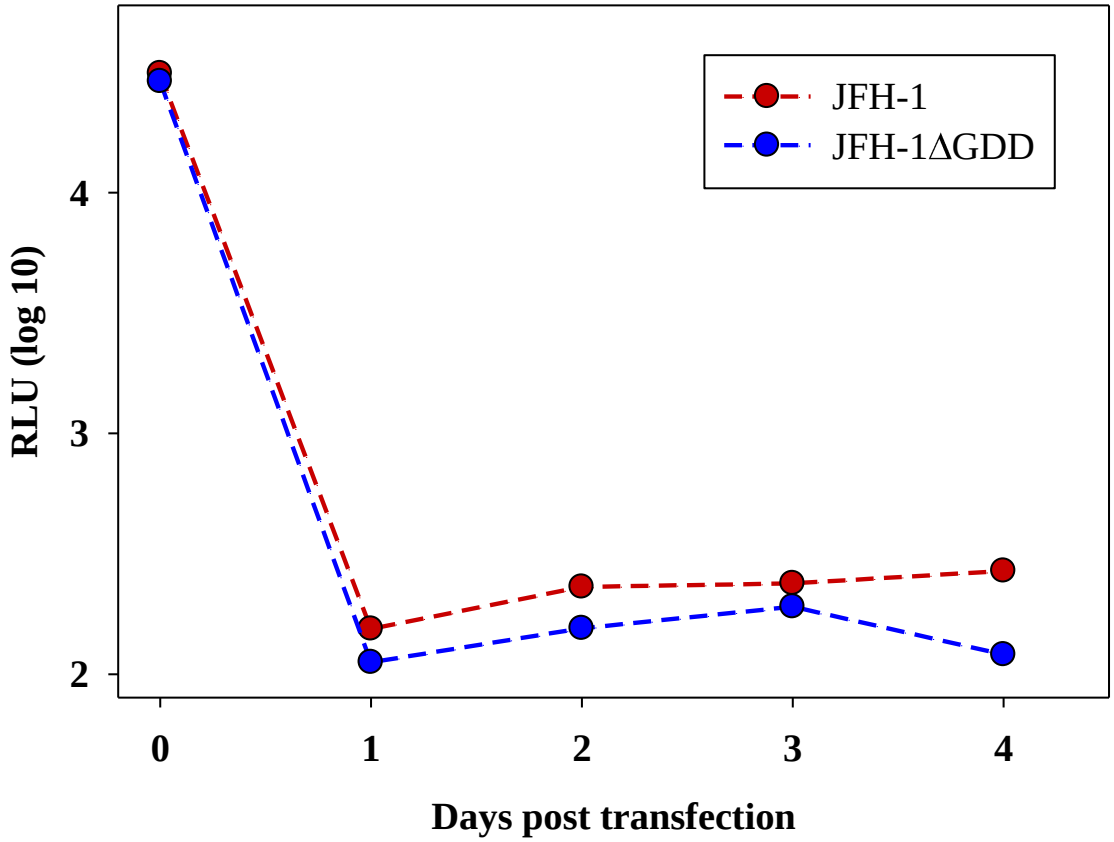

Figure 35: HCV replication in IFNAR deficient primary hepatocytes

Primary hepatocytes isolated from IFNAR knock-out mice were transfected with JFH-1 (red line) or the polymerase deficient JFH-1ΔGDD (blue line) RNA. Luciferase values were measured at 5h post transfection (Day 0) as a read out for transfection efficiency and everyday for the next 4 days.

Luciferase analysis indicative of transfected RNA was detectable 5 hours post transfection. However, the luciferase levels declined one day post transfection and remained undetectable at the time points measured suggesting that only low levels of replication could be maintained in the absence of a type I interferon response.

Again, IFNAR knock-out cells transfected with JFH-1 RNA were cultured in the presence or absence of neutralizing antibodies against type I interferon and luciferase expression indicative of HCV replication was measured.

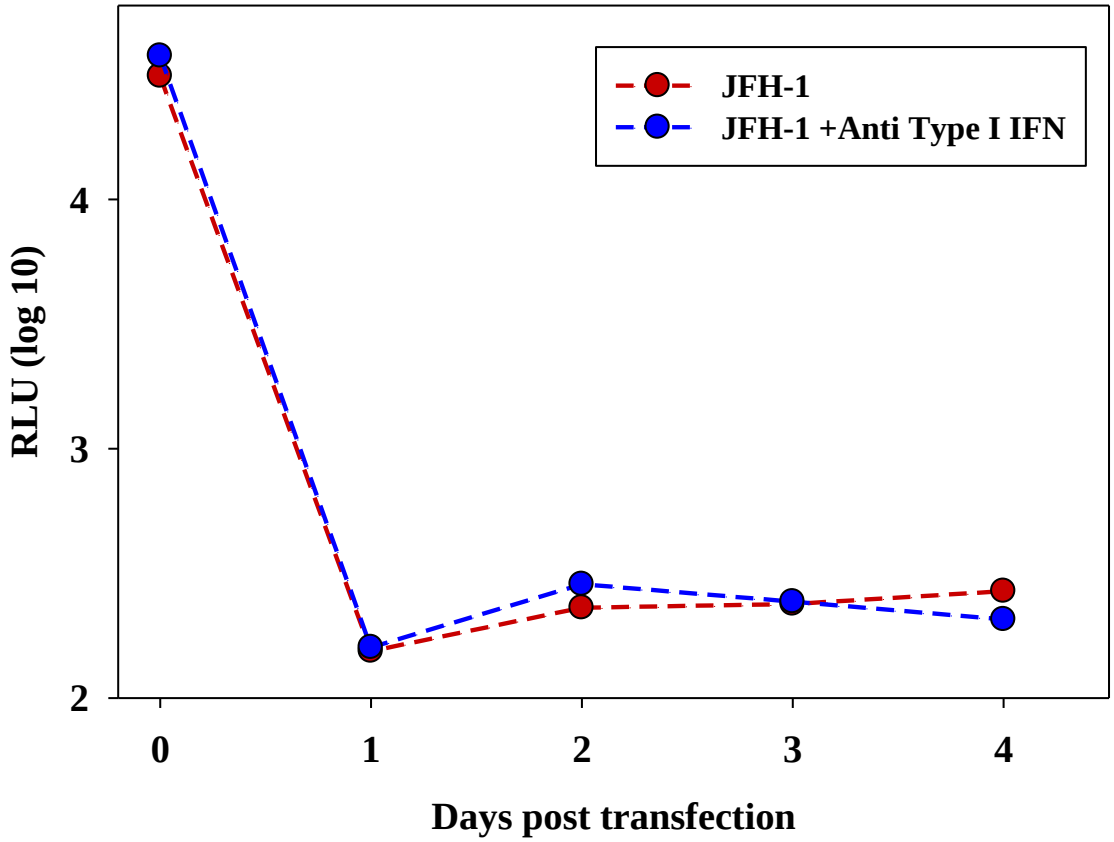

Figure 36: Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in IFNAR knock-out hepatocytes

IFNAR knock-out hepatocytes were transfected with JFH-1 RNA. The electroporated cells were cultured on 12-well plates (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

The presence of neutralizing antibodies to type I interferon did not further increase HCV replication in these cells. This indicates that although HCV replication is blocked in the WT cells, the key restriction factor may not be the type I interferon family. Instead, at this stage it could be hypothesized that in the liver, a type III interferon or the genes induced by this family could be the major inhibitor of HCV replication.

2.4.3 The role of IRF-5, IRF-1 and MAVS in HCV replication inhibition

Since it is known that both the type I and III interferons induce the same set of ISGs, unlike fibroblasts, in addition to type I interferon hepatocytes are also responsive to type III interferon. In order to test the type I IFN independent but type III IFN dependent role of the IRFs, cells defective in key elements of the interferon system were analyzed. To this end,

primary hepatocytes isolated from IRF-5 knock-out mice were analyzed for HCV replication.

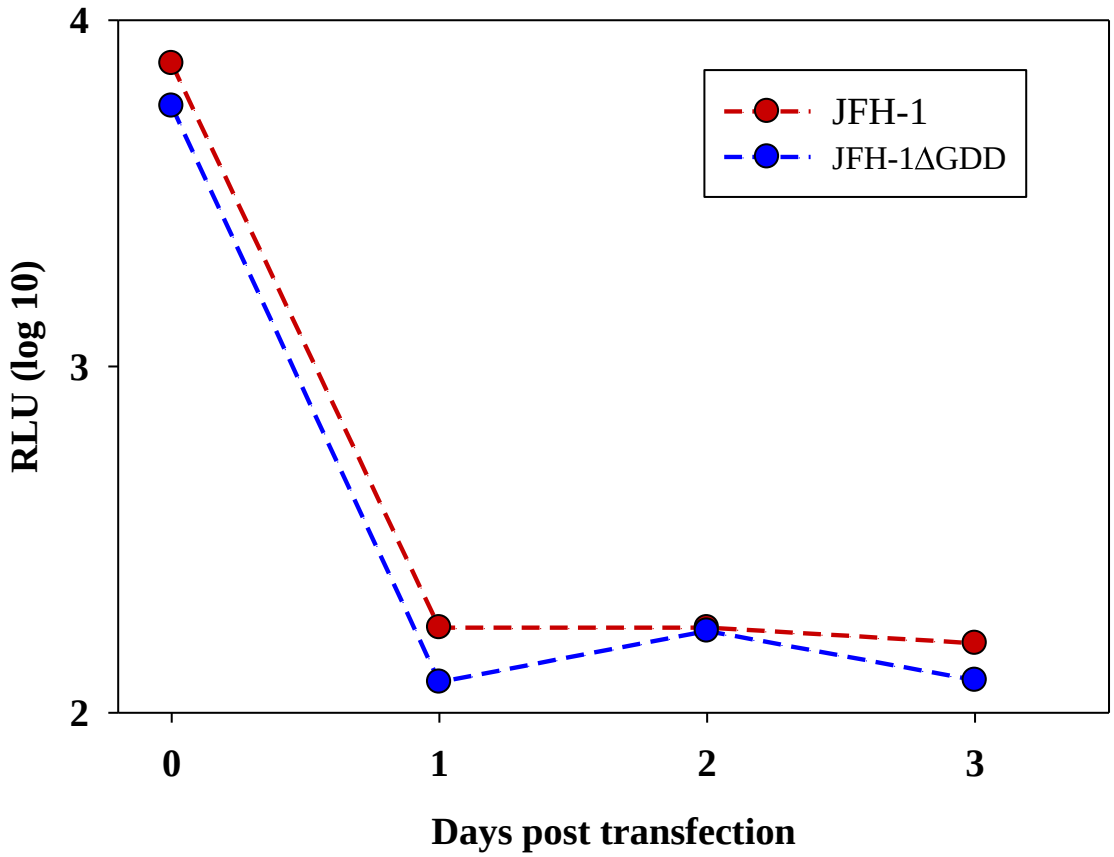

Figure 37: HCV replication in IRF-5 deficient primary hepatocytes

Primary hepatocytes isolated from IRF-5 knock-out mice were transfected with JFH-1 (red line) or the polymerase deficient JFH-1ΔGDD (blue line) RNA. Luciferase values were measured at 5h post transfection (Day 0) as a read out for transfection efficiency and everyday for the next 3 days.

The isolated hepatocytes which were transfected with RNA from the JFH-1 replicon expressed luciferase levels 5 hours post transfection. The level of expression dropped drastically after day 1 to basal levels up to day 3 post transfection. Luciferase levels of polymerase positive replicon was comparable to the polymerase mutant strain (ΔGDD). This suggests that IRF-5 does not play a critical restrictive role in replication of HCV.

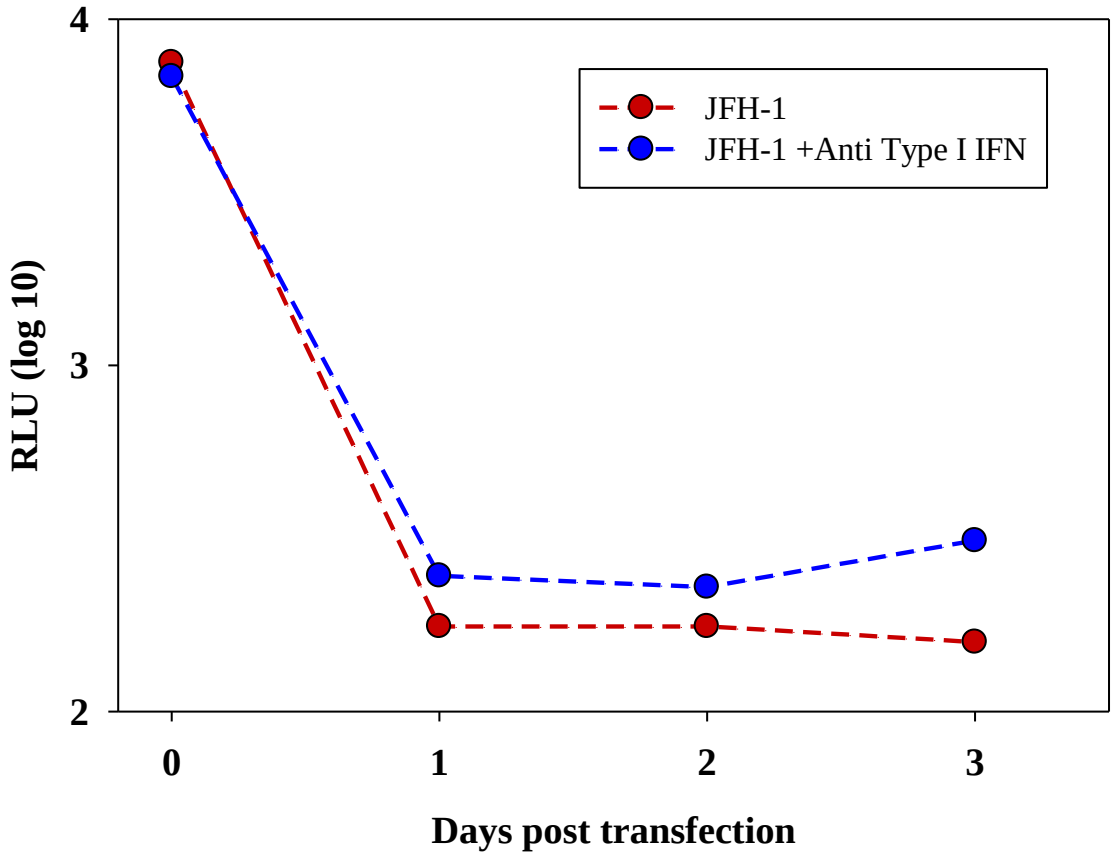

Figure 38: Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in IRF-5 knock-out hepatocytes

IRF-5 knock-out hepatocytes were transfected with JFH-1 RNA. The electroporated cells were cultured on 12-well plates (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

Since the restrictive role of IRF-5 in HCV replication in the absence of an interferon response has been established in mouse embryonic fibroblasts **(Figure 15**) the role of an interferon independent role of IRF-5 in primary heaptocytes was tested. Primary hepatocytes isolated from IRF-5 knock-out mice were transfected with RNA from the JFH-1 replicon and treated in the presence of antibodies against type I interferon and luciferase levels were measured up to day 3 post transfection.

Although the level of luciferase expression dropped after one day post transfection, a slightly higher level of expression was maintained up to day 3 post transfection. The luciferase expression was not as high as that observed in mouse fibroblasts. Additionally, it is also possible that the low transfection efficiency compromised replication levels. Although, direct comparisons between different cell types do not deliver accurate results, these data indicate the possible function of a type I interferon independent response.

Similarly, the role of IRF-1 in HCV clearance has been well elucidated. In order to analyze the role of IRF-1 in HCV replication, primary hepatocytes isolated from IRF-1 knock-out mice were used. Primary hepatocytes isolated from IRF-1 knock-out mice were cultured and transfected with RNA transcribed from JFH-1 subgenoimc replicon or the polymerase mutant JFH-1ΔGDD. Cells were lysed at 5 hours post transfection and everyday for 3 days after that and tested for luciferase expression.

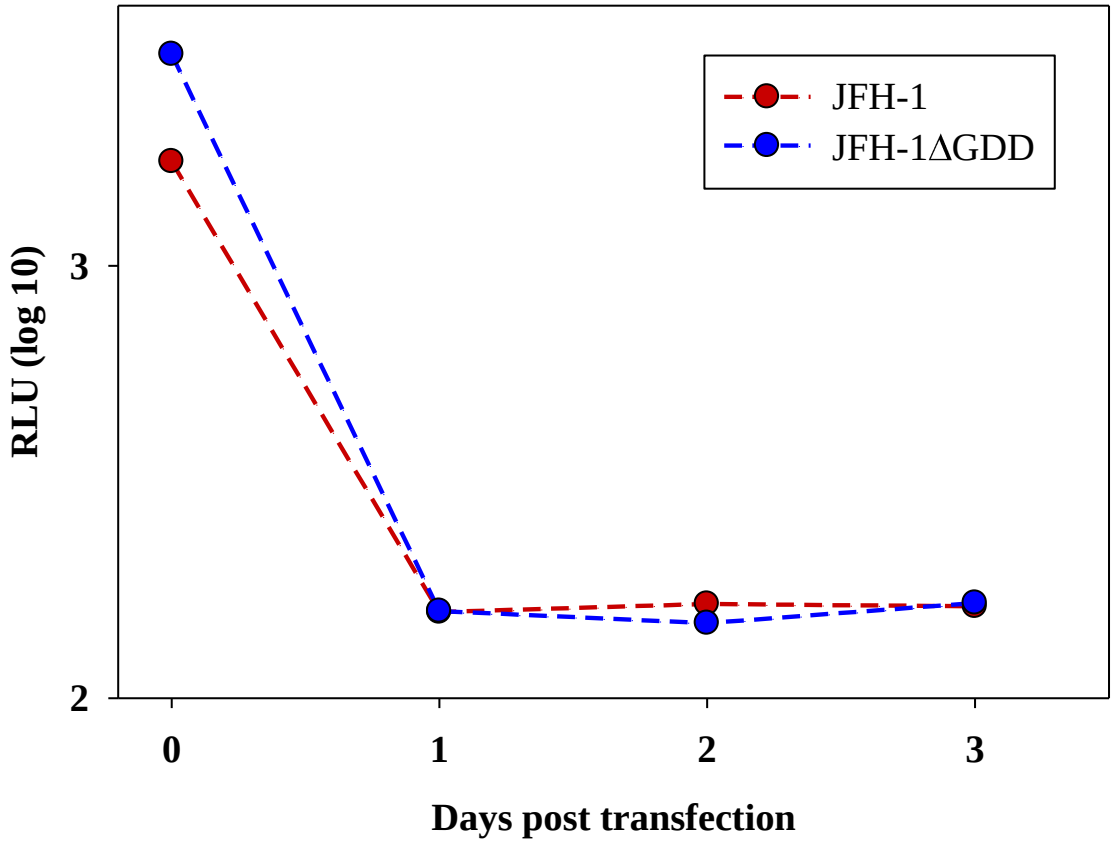

Figure 39: HCV replication in IRF-1 deficient primary hepatocytes

Primary hepatocytes isolated from IRF-1 knock-out mice were transfected with JFH-1 (red line) or the polymerase deficient JFH-1ΔGDD (blue line) RNA. Luciferase values were measured at 5h post transfection (Day 0) as a read out for transfection efficiency and everyday for the next 3 days.

As is shown **Figure 39**, no luciferase expression was observed in the replication competent JFH-1 transfected cells and was comparable to the polymerase mutant JFH-1ΔGDD. This indicates that IRF-1 does not play a restrictive role in HCV replication in mouse hepatocytes.

Since the function of IRF-1 independent of type I interferon in mouse fibroblasts has been elucidated, their role in mouse hepatocytes was analyzed. To this end, IRF-1 knock-out hepatocytes were transfected with JFH-1 subgenoimc replicon and treated with or without antibodies against type I interferon.

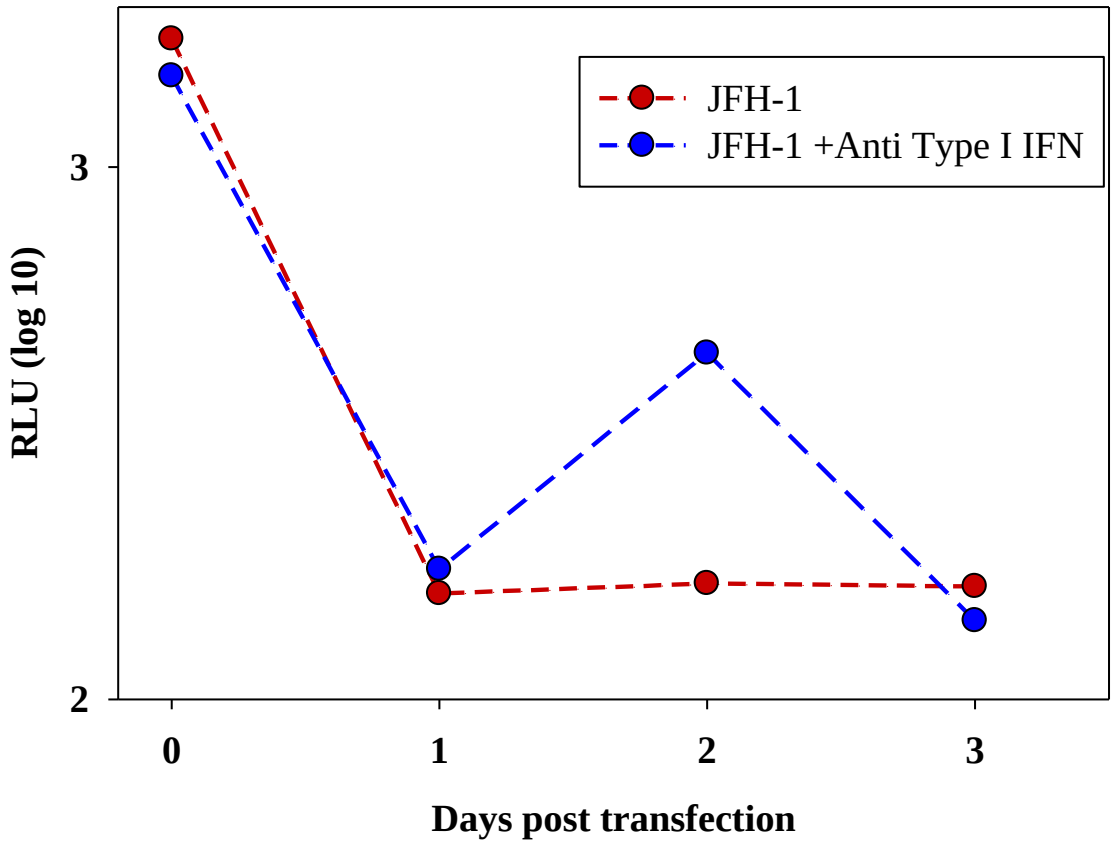

Figure 40: Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in IRF-1 hepatocytes

IRF-1 hepatocytes were transfected with JFH-1 RNA. The electroporated cells were cultured on 12-well plates (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

As shown, there was a slight increase in replication of JFH-1 in the IRF-1 knock-out mice in the absence of a type I interferon response. Replication was only slightly increased in the absence of a type I interferon response. This data indicated a role of a type I interferon independent IRF-1 dependent restrictive function in HCV replication.

Additionally, the role of adaptor protein MAVS has been reported to be essential in maintaining a downstream interferon response[1]. MAVS is a protein localized on the mitochondrial surface and has been reported to be important in the restriction of HCV. In fibroblasts, MAVS has been reported to be an essential component in the IFN-λ pathway. Although IFN-λ is produced by several cell types not all cell components respond to this cytokine[128]. It is understood that both IFN-α and IFN- λ act together to limit viral infection. Cells treated with IFN- λ for a long time and then with IFN- α could completely abrogate viral replication in comparison to cells that were only treated with high levels of IFN- λ[129].

To validate the role of MAVS adaptor protein in HCV replication, primary hepatocytes isolated from MAVS knock-out mice were transfected with *in vitro* transcribed JFH-1 or the polymerase deficient JFH-1ΔGDD RNA. The cells were lysed at 5 hours post transfection as a read-out for transfection efficiency and everyday for the following two days.

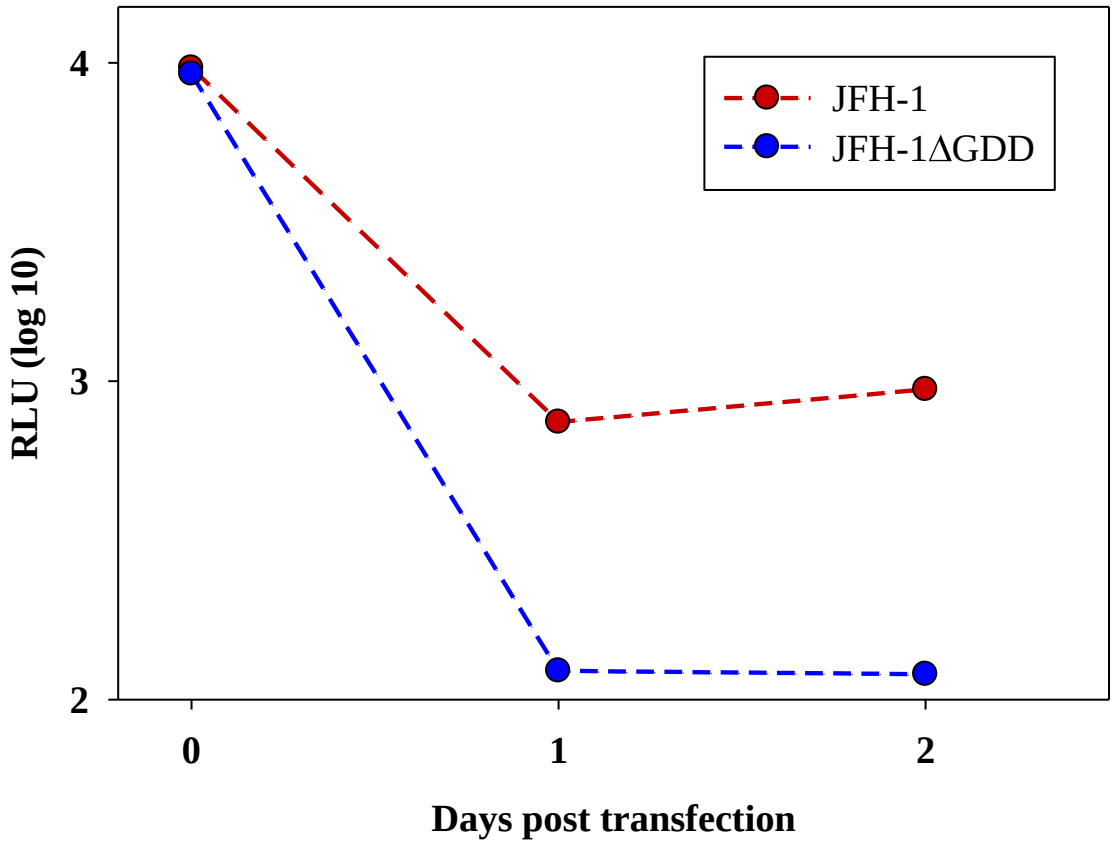

Figure 41: HCV replication in MAVS deficient primary hepatocytes

Primary hepatocytes isolated from MAVS knock-out mice were transfected with JFH-1 (red line) or the polymerase deficient JFH-1ΔGDD (blue line) RNA. Luciferase values were measured at 5h post transfection (Day 0) as a read out for transfection efficiency and everyday for the next 2 days.

As shown in **Figure 41**, luciferase expression could be observed up to day 2 post transfection.

Since MAVS is pivotal in the ensuing interferon response, the role of MAVS in the absence of a type I interferon response in primary hepatocytes was determined. To this end, primary hepatocytes isolated from mice knocked-out for MAVS were transfected with RNA from the polymerase competent HCV JFH-1 strain and cultured in the presence or absence of neutralizing antibodies against type I interferon.

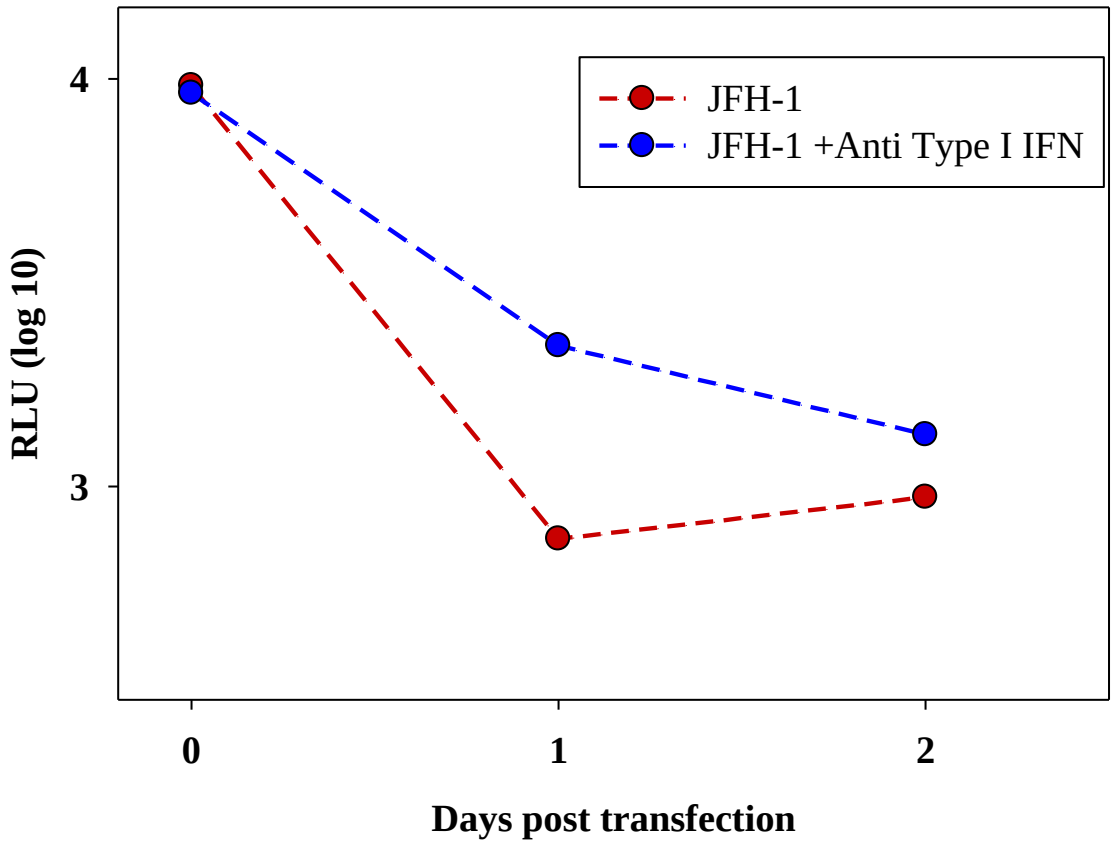

Figure 42: Replication analysis of JFH-1 in the presence of anti-Type I IFN antibodies in MAVS hepatocytes

MAVS hepatocytes were transfected with JFH-1 RNA. The electroporated cells were cultured on 12-well plates (red line) or with (blue line) neutralizing antibodies (2µg/ml) against IFN-α (4EA1) and IFN-β (7FD3).

As was observed a higher level of luciferase expression was observed in cells cultured in the absence of a type I interferon response. This suggests that in hepatocytes, although MAVS was found to be essential in inducing an antiviral response against HCV, the abrogation of an IFN response elucidated a role independent of the type I interferon response.

3. Discussion:

3.1 Inhibitory role of the interferon system against HCV in mouse fibroblasts

The Hepatitis-C is a virus that primarily affects the liver. HCV infects approximately 3% of the global population[11]. Patients chronically infected with the virus often progress to liver fibrosis, cirrhosis and eventually to hepatocellular carcinoma. Currently, there are no vaccines available against HCV, treatment regime is only partly successful and viral clearance is reported to be dependent on several viral and host factors. Studies towards an effective vaccine and treatment are hampered due to the lack of suitable models eg: small animal models. Limited host and tissue tropism of HCV has severely hindered the development of small animal models susceptible to HCV. Currently, the chimpanzee is the only animal model susceptible to HCV infection that can be used to study immune responses against a replicating virus. However, chimpanzee research is expensive, difficult and restricted by ethical constraints. Although HCV is largely hepatotropic, reports indicate the presence of virus particles in the B cells, the cerebrospinal fluid and the endothelium of the blood brain barrier etc[35,37]. Apart from hepatic cells, HCV is also reported to replicate in HEK, HeLa and MEF cells[38-40].

The current treatment regime for HCV is pegylated IFN combined with nucleoside analogue Ribavirin. Although the treatment is largely successful, not all patients achieve the desired sustained virological response[130]. Variations in treatment outcomes have been reported both within and across viral genotypes. Also, within the same genotype, different races of people have shown to respond differently[131]. This implies that although treatment outcome depends on the genotype of the virus, host factors are also responsible for determining response to IFN. This led us to analyze the role of the IFN regulatory factors in limiting HCV replication.

IFN treatment is largely successful in patients and HCV infected cell culture can be ‘cured’ by treating with IFN. Curing cell lines of HCV by treating with IFN is plausibly due to the induction of ISGs. IFN stimulated genes induced by IFN can directly inhibit HCV. However, the mechanism of how these ISGs inhibit HCV is still largely unclear. Viperin is one such ISG that binds host protein hVAP-33 and inhibits HCV from replicating by

inhibiting its interaction with the viral NS5A[132]. Although the direct functions of several other ISGs in the inhibition of HCV are predicted, the exact mechanisms are still elusive.

HCV has been reported to induce the IFN system. RIG-I and TLR3 are pathogen recognition receptors that are adept at recognizing specific pathogen signatures; the 5' triphosphate characteristic to non-host RNA and the double stranded HCV replication intermediate respectively[120,133]. Viral recognition and PRR activation leads to subsequent stimulation of IFN stimulated genes that lead to a cellular antiviral response.

An activated interferon system is a result of several activated intermediate factors that culminate in the transcription of antiviral genes. The interferon regulatory factors are mediators of such an interferon response. In order to elucidate the role of individual IRFs in limiting HCV replication, mouse embryonic fibroblasts deficient in IRFs and other known effectors were transfected with HCV RNA and replication potential was observed.

Interestingly, like most viruses, HCV has developed mechanisms to evade a functional IFN response. The structural and non-structural proteins of HCV have been reported to inhibit HCV replication by cleavage of the adaptor proteins MAVS[1] and TRIF[2] blunting the RIG-I and TLR3 mediated immune responses. Additionally, STAT1 proteins important in amplifying the IFN response are effectively counteracted by the HCV proteins.

Replication studies of HCV are largely performed on human hepatoma cells such as Huh7 and its derivative Huh7.5. Hepatoma derived cell lines are both susceptible and permissive to HCV and have proven to be valuable cellular targets for replication and infection studies. Due to a mutation of RIG-I in Huh7.5[118] and an added down regulation of TRL3 in Huh7 cells[2], immune response towards HCV is limited to pathways excluding TLR3 in Huh7 and RIG-I and TLR3 in Huh7.5 cells. These lesions in the IFN pathway could be the reason for HCV permissivity.

Although mouse cells are not naturally susceptible or permissive to HCV infection, replication has been observed in cell lines with a defective IFN system[40]. Since MEFs have a fully functional and competent IFN signalling system, transfection of HCV replicons or subgenoimc replicons into mouse embryonic fibroblasts enables the detection of host responses against the virus. Additionally, the availability of mouse embryonic fibroblasts with specific lesions in the proteins of the IFN system provides the opportunity to study their individual roles in restricting HCV replication. Conditionally immortalized fibroblasts allow convenient maintenance of cells in culture while transforming to a 'primary like' phenotype in the absence of permissive conditions. The results described in this study are based on the subgenoimc replicon that codes only for a part of the viral genome. Viral

infection studies require full genome expression and are not covered in this study owing to the additional requirement of entry receptors on the surface of mouse cells.

HCV replication is reported to be enhanced in the presence of microRNA-122[40]. Liver specific miR-122 reportedly enhances the conformation of the homologous IRES element aiding in translation. MiR-122 also increases replication of HCV although the mechanism by which it does so is still elusive. Therefore, mouse embryonic fibroblasts expressing miR-122 permit enhanced replication and more importantly allow analysis of the IFN response directed against the viral genome.

HCV isolates from patients replicate weakly, if at all in cell culture[134]. This led to the discovery of the replicon technology[78]. This system essentially codes for the viral genome along with the acquired mutations that enable replication and virus production in cell culture. The replicon system has been useful in studying the viral life cycle as well as in drug screening. However, replicons are available only for genotypes 1b and 2a and not for the other recognized genotypes limiting research to the use of only these strains.

In this study, primary-like cells were used to investigate the restricting role of IFN-dependent and IFN-independent mechanisms to HCV replication.

To determine the role of the type I IFN system in the restriction of HCV replication we compared the replication of a subgenoimc genotype 2a strain (JFH1) luciferase replicon in the different knock-out fibroblasts. As observed in **Figure 7**, WT MEFs could not maintain continued replication, indicating restriction factors in MEFs with prohibit HCV replication in MEFs.

In contrast, MEFs with a defective IFN response (ie: with a non-functional type I IFN receptor) were incapable of a continued antiviral response with leads to HCV replication (**Figure 9**). This suggests that the IFN system is component in limiting HCV replication and indicates that restriction of HCV replication in WT MEFs was a result of a fully competent IFN system and not due to the absence of exclusive host factors. However, the role of such complementary factors in viral packaging, assembly and release may be important but are not studied here.

IFN regulatory factors are transcription factors pivotal in relaying activation signals from cellular pattern recognition receptors to the nucleus and inducing an effective antiviral response. IRF-3, an important mediator of antiviral defence works downstream of the MAVS and TRIF mediated RIG-I and TLR3 signalling and directly binds to the promoter of IFNα4 and IFN-β genes as well as a subset of ISGs. In the absence of IRF-3, induction of an antiviral response mediated by RIG-I and TLR3 are inhibited. This lesion stunts the

IFN response significantly resulting in a blunted antiviral response. Deletion of IRF-3 in MEFs permitted replication of HCV, suggesting that IRF-3 plays an important restrictive role in HCV replication in mouse fibroblasts. Blocking of the IFN response pathway in these MEFs did not further enhance replication. IRF-3 can direct antiviral responses by either inducing ISGs, or by stimulating an IFN response. The depletion of type I IFN responses did not enhance replication suggesting that the type I IFN response plays no role in HCV inhibition. This implies that the direct ISG induction is sufficient to limit HCV replication. Additionally, the already high level of replication probably inhibits further replication. This indicates that the latent IRF-3 undergoes activation and results in an antiviral response and this response is independent of IFN amplification.

IFN induced upon viral challenge binds to the IFN receptor inducing the transcription of antiviral genes such as IRF-7, which upon activation is responsible for the induction of the late IFN-αs and thereby the amplification of the IFN response. Experiments performed on MEFs deficient in IRF-7 exhibited no replication of HCV (**Figure 13**). The results indicate that the amplification of the type I IFN response by IRF-7 was not responsible to limit HCV replication. Therefore, IRF-7 may not be an important factor in limiting HCV replication.

Taken together, the inability of WT and IRF-$7^{-/-}$ MEFs to maintain replication indicates that the response to IFN has little effect on limiting HCV replication. However, inhibiting the IFN response in IRF-$7^{-/-}$ MEFs resulted in a significant increase in HCV replication (**Figure 27)**. The blunted IFN response along with the added lesion of IRF-7 permits the replication of HCV.

Similarly, the role of IRF-5 in immune function is well established. In this study, mouse fibroblasts deficient in IRF-5 could not maintain HCV replication (**Figure 15**). This suggests that although IRF-5 is pivotal in antiviral defence, it is dispensable to HCV replication in mouse fibroblasts. The IRF-5 independent antiviral protection could be explained as a result of cellular immune responses induced by other IRFs and the subsequently induced ISGs.

In addition, inhibition of the IFN response in IRF-$5^{-/-}$ MEFs resulted in a sustained and detectable luciferase expression indicative of ongoing HCV replication. The data indicate that in the absence of an IFN response, the deletion of IRF-5 dampens the immune responses against HCV resulting in HCV replication.

Since viruses have evolved to antagonize the IFN pathway, the antiviral pathways induced by IRFs may lead to the induction of antiviral genes. IRF-1 is involved in antiviral defence

and its role as an IFN-independent antiviral strategy has been well elucidated[5,116]. The SNP (-300AA) in the *IRF-1* gene lead to a higher inducibiltiy of the gene and has been associated with better response to treatment in patients infected with genotype 1[115]. In addition, over-expression of IRF-1 inhibits HCV replication *in vitro*, indicating a critical role of IRF-1 in limiting HCV replication. However, MEFs lacking IRF-1 did not support HCV replication (**Figure 16)**. A combined defect in IFN response as well as IRF-1 resulted in a significant increase in HCV replication (**Figure 28**). Therefore, in the absence of a functional IFN signalling, IRF-1 may have a potentially restrictive function in HCV replication. Interestingly, patients who develop auto antibodies against IFNs do not attain sustained virological response[135]. Mutated or dysfunctional IRF-1 in patients non-responsive to IFN may be reason for viral persistence.

The functional relevance of MAVS protein as an adaptor molecule in IFN signalling through the RIG-I and TLR3 pathways is well established[1]. NS3/4A protein of HCV mediated cleavage of human MAVS protein by HCV to circumvent the induction of the IFN response has been well characterized[1]. However, deletion of MAVS in mouse fibroblasts was not sufficient to permit HCV replication (**Figure 19**). Again, this indicates that although the RIG-I dependent signalling network was blunted, the TLR-3 mediated pathway functioning through TRIF was still functional and is probably sufficient to restrict HCV replication.

Additionally, IRF-1[5,123] and IRF-5[136] are known to be responsible for stimulation of ISGs independent of IFN signals.

Additionally, protein kinase R (PKR) has been reported to be pivotal to antiviral defence. Following binding to dsRNA, PKR dimerizes and undergoes autophosphorylation. Thus activated, PKR phosphorylates eukaryotic initiation factor 2 eIF2A suppressing the cellular translation machinery[137-139]. In order to circumvent the lack of the host translational machinery, HCV has developed a functional IRES element. The IRES element aids in ribosome-mediated translation of the viral proteins. This suggests that PKR functions in inhibiting HCV replication in a manner independent of the type I IFN response. Because PKR expression is dependent on type I IFN, depletion of constitutive levels of type I IFN could reduce PKR expression and therefore PKR dependent restriction of HCV replication.

Apart from the IRFs, IFN stimulated genes have been reported to be pivotal in anti HCV defence. STAT1 is a protein that acts downstream of the IFN receptor and is responsible for IFN amplification. This complex translocates to the nucleus and induces genes responsive to STAT1 binding. Additionally, STAT1 has been shown to be important in

restricting HCV replication[140]. Therefore it is not surprising that HCV has evolved to block the STAT1 activation pathway and the subsequent downstream signalling. The core protein of HCV has been shown to associate with STAT1 and induce degradation in a proteasome-dependent manner[113]. Additionally, the HCV NS5A has been observed to inhibit STAT1 phosphorylation, suppressing nuclear translocation and the ensuing IFN response[3,113]. Replication analysis of HCV in STAT1 knock out MEFs showed detectable levels of luciferase expression indicating that a lesion in STAT1 is sufficient to maintain HCV replication in these cells (**Figure 12**).

HCV RNA harbours specific structures which are recognized by the cells as PAMPs and results in the induction of a type I IFN. Induction of IFNs were observed in MEFs transfected with the JFH-1ΔGDD RNA, but the polymerase competent JFH-1 RNA was observed to induce significantly higher levels of IFN. This observation was plausibly due to the double stranded replication intermediates[63] or due to the higher levels of RNA as a result of ongoing replication.

A higher induction of IFN was observed in WT MEFs as compared to all other transfected cell types (**Figure 20**). This could be the plausible reason why HCV could not replicate in these cells. These data suggest that replication must occur at the very early stages in WT cells, but the induced IFN response is sufficient to inhibit replication. This could explain the lack of replication in WT MEFs in spite of the deleted IFN response. Cell lines such as IFNAR and IRF-7 induce lower amounts of IFN (**Figure 21**) which could be due to the absence of the IFN amplification loop. The role of type I and III IFNs have been shown to be important in inhibiting HCV. Type III IFN/ IFN- lambda has been shown to have inhibitory effects on HCV in human hepatoma cells[141]. IL28B is associated to viral resistance and is known to be upregulated by IFNs and by HCV infection[101]. Two SNPs located near the gene region encoding IFN-λ3 (rs12980275 and rs8099917) in HCV infected patients treated with the combination therapy has been associated to treatment outcome[101,102].

However, in MEFs, it was observed that HCV restriction is solely dependent on the type I IFN because IFN response could be completely blocked by anti type I IFN (**Figure 21**).

Since, type I IFN were observed to be induced in MEFs transfected with HCV the role of these individual factors independent of the IFN response was analyzed. To this end, WT and IFNAR knock out MEFs were electroporated with RNA transcribed from the JFH-1 strain and were cultured in the presence or absence of neutralizing antibodies against type I IFN. In the absence of an IFN amplification loop, no HCV replication was observed in WT

MEFs suggesting that the competent endogenous IFN levels and ISGs in the cell was sufficient to limit HCV replication. No additional replication was observed in the IFNAR knock out MEFs.

WT cells induce higher amounts of IFN in response to HCV than IFNAR and IRF-7, which could be due to the lack of amplification loop in the absence of IFNAR and IRF-7. The higher amounts of IFN secreted by WT MEFs in response to HCV could be the reason for the inhibition of HCV replication. In order to test this, replication assays were performed in the presence of depleting antibodies to type I IFN. WT cells permitted no replication of HCV indicating that other mechanisms were important.

However, depletion of type I IFN in IRF-1, IRF-5 and IRF-7 deficient cell lines revealed a significantly high replication level indicating the important restrictive functions of these factors in HCV replication inhibition. Replication levels were also higher in PKR and MAVS deficient MEFs.

Taken together, the inability of WT MEFs to maintain replication indicates that in the WT MEFs, the response to IFN has little effect on limiting HCV replication. The ability of IFNAR knock out MEFs to support HCV replication could be attributed to plausibly low levels of endogenous IFN and the subsequent decrease in IFN stimulated gene expression.

The absence of individual proteins IFNAR, IRF-3, PKR and STAT1 were shown to enhance HCV replication. However, IRF-1, IRF-5 and IRF-7 were dispensable in limiting HCV replication. Surprisingly, the loss of IRF-1, IRF-5 and IRF-7 in MEFs in the absence of a type I IFN response resulted in significantly higher expression of luciferase indicative of active replication. These data indicate the additional roles of IRF-1, IRF-5 and IRF-7 in limiting HCV replication in the absence of IFN response.

3.2 Inducible cell lines for analyzing virus-host interactions at very early time points

HCV is a poor inducer of IFN, an aspect that can be attributed to the many ways the virus has evolved to evade the host IFN machinery. HCV viral proteins have evolved techniques to either cleave[1,2] or successfully escape[3] host proteins essential in the induction of IFN responses. Evidence from studies on experimentally infected chimpanzees suggests that the time point and kinetics of ISG induction could help determine disease outcome, chimpanzees which show high ISG expression levels at early time points of infections clear HCV infection more efficient whereas a later ISG response is associated with development of chronic HCV[100]. This indicates that the detection of ISGs induced at very early time points can prove to be markers for progression to chronic infection or the resolving of infection at the acute stage itself. Thus, the it is important to study such responses in cell lines at very early time points. To this end, the inducible HCV replicon was generated along with a separate polymerase mutant plasmid that serves as a replication deficient control. The novelty of these plasmids lies in the fact that when single cell clones are generated, not only can the transcription be controlled exogenously but the cellular response is largely synchronous. This advantage is of great importance in the detection of genes only mildly induced that are otherwise lost in transient transfections where only a population of cells are transfected. Since the inducibility of these plasmids has been elucidated, it is evident that a technology wherein HCV can undergo controlled transcription has been generated. Additionally, the ability of the polymerase competent plasmid to replicate using the viral NS5B RNA dependent RNA polymerase (RdRp) allows the virus to replicate in culture. This is of utmost importance as viral replication and the double stranded intermediate formed in the process have been reported to induce IFN stimulated genes[133]. The comparison of the replicating RNA to the polymerase negative control will provide us information about the IFN stimulated genes induced upon replication.

Taken together, this system is an excellent tool to analyze the virus-host interaction with time.

3.3 Inhibitory role of the IFN system against HCV in mouse hepatocytes

Until recently much of HCV research was carried out on hepatoma cell lines which have known lesions in the receptors of IFN signalling. Owing to this defect, observations made on these cell lies do not provide an accurate representation of virus host interactions and responses thereby. To circumvent this, human fetal liver cells (HFLC) as well as primary human hepatocytes (PHH) have been used for investigations on host-virus interactions.

Since it was identified using fibroblasts that a strong IFN system could effectively block HCV replication; the role of the type I IFN as the dominant restriction factor for HCV replication in primary hepatocytes was analysed. HCV subgenomic replicons are not able to replicate in primary hepatocytes isolated from WT mice. In addition, neutralization of type I IFN does not allow HCV replication. Surprisingly, IFNAR-/- MEFs allow replication of HCV RNA, may be due to a lower basal expression of antiviral genes in IFNAR-/- cells. To test if this hypothesis is true for primary hepatocytes, hepatocytes isolated from mice defective in the type I IFN response were transfected and replication potential in these cells were analyzed. An insignificant rise in replication was observed in these cells (**Figure 35**) suggesting that in contrast to MEFs, replication inhibition of HCV in hepatocytes was not mainly due to the type I IFN response. This indicates that the restrictive mechanisms working against HCV in hepatocytes have a low dependence on type I IFN. It has been reported that in spite of the separate, distinct receptor that IFN-λ uses, pegylated IFN-λ was capable of inducing an identical antiviral response as type I IFN[142]. This is further corroborated by the fact that IFN-λ is currently in clinical trials for HCV clearance.

The type III IFN family is the most recent of the identified IFN families. Type I and III interferons induce a similar set of ISGs[142], probably due to the induction of similar STATs[143]. Unlike the type I IFN that all nucleated cells can secrete and respond to, the type III IFN or the IFN-λ family show tissue specific responses. The type III IFN family has been shown to be largely epithelial cell specific[144]. In addition to being differentially induced, it is also implied that type III IFN signalling may be resistant to viral feedback mechanisms targeting type I IFN loops[145].

The IL28B locus has been reported to be an important marker for disease treatment outcome[101-103]. Why and how patients with a particular IL28B SNP clear the virus better is

still elusive. A detectable level of ISG induction is reported in the livers of patients with acute and chronic HCV although detection of IFN-β may not be detected[65,146].

It was observed that hepatocytes isolated from WT mice with a healthy IFN signalling system did not permit HCV replication (**Figure 33**). Additionally, blocking of the type I IFN response did not enhance replication and the luciferase levels indicative of replicating virus remained undetectable (**Figure 34**). These data suggest that replication must occur at the very early stages in WT cells, but the induced IFN response was sufficient to inhibit replication.This suggested that a strong IFN system from the host was inhibiting replication of HCV.

In addition to type I IFN, MAVS has been shown to regulate IFN-λ genes[147], therefore it could be hypothesized that the antiviral responses directed against HCV may share the same regulatory factors as type I IFN. In line with this data, hepatocytes with a defective MAVS protein seemed to maintain HCV replication (**Figure 41**). This phenotype was exaggerated when the type I IFN response was ablated (**Figure 42**). Replication of HCV observed in MAVS knock-out hepatocytes suggest that MAVS is pivotal in anti HCV defence and increased replication in the absence of a type I IFN response suggests a type I IFN independent function of MAVS protein. Since MAVS has been shown to regulate IFN-λ genes[147], it is not surprising that elevated replication levels could be observed in MAVS deficient cells. Additionally, in the context of VSV, a novel dependence on MAVS localization was studied. Peroxisomal MAVS were observed to induce interferon-independent antiviral factors that provide immediate but short-lived protection whereas, the mitochondrial MAVS paved way for a delayed but longer antiviral response[123]. Since IFN-λ has been shown to induce a similar set of ISGs as type I IFN[141,142], and since IFN-λ activates STATs similar to IFN-α/β *in vitro,* the potential role of individual IRFs in viral clearance was studied. Replication assays performed on IRF-1 knock-out hepatocytes revealed that IRF-1 did not play an important role in limiting HCV replication (**Figure 39**). Also, in the absence of a type I IFN response an increase in replication was observed (**Figure 40**). Since IRF-1 has been indicated to be important in HCV restriction, studies on hepatocytes with deleted IRF-1 and MAVS (double knock-out) protein could further elucidate the importance of these factors in viral restriction.

Similarly, replication assays performed on hepatocytes from IRF-5 knock-out mice revealed that IRF-5 did not play a pivotal role in restricting HCV replication (**Figure 37**). However, a slight effect of IRF-5 was observed in the absence of a type I IFN response (**Figure 38**) suggesting a type I IFN-independent restrictive function in HCV replication.

Taken together, the data indicate that HCV replication in mouse hepatocytes is restricted by a strong IFN response largely independent of the type I IFN response. Therefore, this could indicate the presence of a dominant type III IFN response against HCV in hepatocytes. Whether type III IFN dependent or independent factors act as dominant inhibition factors in HCV restriction will have been studied in the context of a depleted type III IFN response.

These data indicate that although responses dependent and independent of type I IFNs are pivotal in antiviral defence in fibroblasts cell lines, in the hepatocytes the type III IFNs may play an important restrictive role.

In line with these findings, recent reports on experimentally infected chimpanzees showed that type III IFNs but not type I IFNs mediated ISG induction and antiviral response in hepatocytes[70]. Considering the similar sets of ISGs induced by both type I and III IFN families[141,142], the reason behind a strong type III IFN response could be the stimulation of an antiviral response in the event of an impaired type I response mediated by viruses.

Additionally, it is also argued that like type I IFN, IFN-λ could be important in differentiation and maturation of DCs.

4. Outlook

The function of the IFN system has proven to be indispensible in limiting Hepatitis-C virus infection. This thesis identifies the importance of the IFN regulatory factors and IFN stimulated genes in restricting replication of HCV. Since mouse cells are not susceptible to HCV infection, only factors restricting replication of the subgenoimc replicon could be identified with this experimental set-up. The host system has been reported to be adept at restricting entry as well as budding and release of virus particles. In order to identify host factors restricting these crucial stages, mouse cells must be made susceptible to infection. The entry receptors required for HCV infection have been identified as scavenger receptor class B member protein SR-B1, tight junction proteins occludin and claudin as well as CD-81, a member of the tetraspanin family of proteins. Although the murine versions of claudin and SR-B1 are functional, mouse cells have to be complemented with the human versions of CD-81 and occludin to successfully aid in HCV entry.

Expression of these entry factors in mouse cells can serve as an infection model enabling the elucidation of an IFN response against HCV. With this model, additional restrictive roles of the IFN regulatory factors or IFN stimulate genes on the entry or budding and viral release can be defined. The determination of host factors aiding in limiting HCV replication by induction of a strong IFN response could lead to increased understanding of response to treatment and disease outcome.

The analyses of IFN stimulated genes in the established stable replication competent cell lines expressing HCV can be determined. An assessment of gene induction at the very early time points can also be determined.

5. Material and Methods:

5.1 Chemicals

The chemicals used for the purpose of this study were supplied by the following companies: Amersham Biosciences, Bayer, Bioline, BioRad, BRL Difco, Gibco, Merck, Promega, PAA, Qiagen, R&D systems, Roche, Seromed, Serva and Sigma.

Enzymes were purchased from Bioline, Invitrogen, New England Biolabs (NEB), Promega, Roche and oligonucleotides were synthesized at Eurofins MWG Operon.

Antibodies were purchased from eBioscience, Pharmingen and Beckton Dickenson.

5.2 Consumables

Table 1 :List of Consumables -

Article	**Company**
Cell culture plates(24 well,12 well, 6 well)	Nunc, Corning,
Tissue culture dishes	Corning, Greiner Bio-one
Reaction tubes (15ml, 50ml)	Greiner Bio-one
Flow cytometry tubes	Sarstedt
Tissue culture flasks(25cm2 ,75cm2 ,125cm2)	Corning, Greiner Bio-one
Combi tips	Eppendorf
Safe lock tubes	Eppendorf
PCR tubes	Kisker Biotech, Steinfurt
Cryovials	Corning

5.3 Equipment

Table 2: List of Equipments -

Article	**Company**
Analytical balance	Sartorius Model 120S / 500G
Autoclave	Belimed Dampsterilizer 6-6-6 HS1,FD Tecnomara Table-top autoclave Technoclav 50

CCD-Camera	Nikon Coolpix 4500
Cell counter	Schärfe System Casy 1 DT
Cell Sorter	FACS Aria, Becton Dickinson
Cooling centrifuge	Sorvall super speed RC5-C
Deionized water supply	Millipore Milli-Q
Electroporator	Gene Pulser Xcell, BioRad
Freezer	-20 °C: Liebherr Model GS3183 -80 °C: GFL 6485
Fridge	Liebherr Model UKS3600
Incubator for cell culture	Forma Scientific Model 3336 Labotec C200
Shaker	Heidolph REAX3
Light cycler	Roche
LSR II	Becton Dickinson
Microscope	Leitz Labovert FS Olympus Type CKX41 und CK2
Microwave	Whirlpool Model Pro 825
Mini centrifuge	Heraeus-Christ, Biofuge fresco
Nitrogen tank	HarscoK-Series Modell 17K
PCR machine	Biometra T3 Thermocycler
pH-Meter	Beckmann M340
Photometer	Thermo Electron Corporation, Multiskan EX
Pipettes	Gilson Pipetman Labnet Biopette
Pipettor	Pipetboy IBS Integra Biosciences
Power Pack	Biorad Power Pac 300
Refrigerated centrifuge	Juan ModellCR412
Spectral photometer	PeqLab Nanodrop ND-1000
Table centrifuge	Heraeus Biofuge pico Heraeus Sepatech Megafuge 1.0R
Thermo shaker	Eppendorf Model compact
Vortex	Heidolph REAX 2000
Ultra centrifuge	Sorval Combi OTDC; rotor Beckman T150, T10
Water bath	Rowa Model Ro 3044

5.4 Software

This thesis was written using the Microsoft (MS) Word 97-2003 version. Tables, calculations and graphs were constructed on MS Excel 97-2003 as well as Graph pad PRISM and Sigma Plot version 11. Figure design and annotation was performed with MS PowerPoint 97-2003. Microscopical image analysis was performed with ImageJ (National Institutes of Health) and the corresponding plugins as well as Zeiss LSM510 software. Flow cytometry data was acquired and analyzed with BD CellQuest, BD FACSDiva and FlowJo v7.6 . Vector NTI Advance 4/10/11 was used for in-silico cloning, sequence alignment and primer design. Primer3 was used for primer design. Sequencing results were analyzed with Chromas version 2.32 (Technelysium Pty Ltd. Quantitative PCR (qPCR) results were analyzed and calculated using the LightCycler 480 software.

5.5 Sterilization

Glassware was sterilized at 180˚C for 4 hours prior to use. Plastic materials such as Eppendorf tubes, pipette tips and solutions for cell culture were autoclaved at 121˚C for 25 minutes. Solutions that could not be autoclaved were filtered through a 0.22μm filter under a clean working bench.

5.6 Photometric determination of nucleic acid concentration

Concentration of nucleic acids was assessed by measuring absorbance at A260 on a Nanodrop-1000. Purity of nucleic acids preparation was determined by the A260/280 ratio. A value appreciably lower than 1.8 and 2 for DNA and RNA respectively indicates a contamination due to protein, phenol or organic compounds in the sample.

5.7 *In vitro* transcription and RNA preparation

For elctroporation, plasmid DNA was linearized, *in vitro* transcribed and purified. To this end, 10ug each of pJFH1 and pJFH1ΔGDD were linearized using Mlu1 restriction enzyme (NEB) at 37˚C for 1 hour. The linearized DNA was extracted in the presence of 1/10 volume of 3M sodium acetate (pH5.4) and 200ul of cold phenol equilibrated with TE. Upon centrifugation, at 13500rpm for 3 min the supernatant was extracted and the step was repeated. Following the extraction of the supernatant in the first tube 200ul of ddH2o was added and subjected to second round of centrifugation in order to extract any DNA still

remaining. Finally, an equal volume of chloroform was added to the supernatant and centrifuged again at the same conditions. Again, the supernatant was separated and 2.5 volumes of 100% ethanol was added and stored at -20˚C for 1 hour or overnight. After this, the tube was centrifuged at 13500rpm for 20 minutes at 4C. The DNA pellet was then washed with 80% ethanol and dried briefly at 37˚C. The ethanol-free pellet was dissolved in 50ul RNAse-free water. The concentration of linearized DNA was quantified using the Nanpdrop.

The linearized DNA was subsequently subjected to *in vitro* transcription in the presence of 5X RRL buffer (400mM HEPES pH 7.5; 60mM MgCl2, 10mM Spermidine, 200mM DTT), 25mM rNTP, 40U/µl RNAse inhibitor, and 80U T7 polymerase and incubated for 2hours at 37C. Following this, 2U of T7 polymerase was added additionally and incubated again for 2hours at 37C. Finally, 2U/µg of DNAse was added and incubated for 30 minutes at 37C.

The RNA thus transcribed was extracted using 2M sodium actetate (pH 4.5) in the presence of water and aqua saturated phenol (pH<5). The mixture was vortexed and incubated on ice for 10 minutes. The reaction mixture was then centrifuged at 13500rpm for 10 minutes at 4C. The supernatant obtained following centrifugation was mixed with equal volume of chloroform and subjected to another round of centrifugation for 3minutes at 13500rpm. RNA was precipitated from the supernatant by adding 0.7 volumes of isopropanol equilibrated to room temperature. Finally, the mixture was centrifuged at 13500rpm for 15minutes at room temperature and the pellet thus obtained was washed once with 70% ethanol. Once briefly air dried, the pellet was resuspended in 50ul RNAse free water and the concentration measured on the Nanodrop.

5.8 Isolation of murine cells

5.8.1 Mouse embryonic fibroblasts

MEFs were isolated from mice E13.5-14.5 post coitus. During this stage of gestation, the developing organs are coalesced making it easier to discard. E13.5 embryos were decapitated and the heart, lungs and abdominal organs were scooped out and the remaining mesenchyme surrounding the somites which is the source of most fibroblasts was obtained by digesting in Trypsin for 30minutes at 37˚C and plating in DMEM 3+ as mentioned above.

5.8.2 Primary mouse hepatocytes

To obtain fresh mouse primary hepatocytes, 6-8 week old male mice were anesthetized with 0.1ml/10g body mass of ketamin(10%) xylazine(5%) NaCl(85%). Upon sedation, an incision was made on the skin exposing the abdominal region. The peritoneum was briefly rinsed with 70% ethanol following which it was cut open to expose the liver and the abdominal region. The intestines were carefully shifted to the side exposing the hepatic portal vein and the inferior vena cava. An intravenous canula was carefully inserted into the vena cava and the liver perfusion media supplemented with heparin (500U/ml) was injected at a flow rate of 8ml/min to flush out all non-resident liver cells. Liver digest media supplemented with collangenase (55mg/150ml) was allowed to flow at a constant flow rate of 25ml/min at a temperature of 37˚C. The digested liver was excised and hepatocytes were gently released from the liver. Liberated cells in suspension were then separated from any remaining undigested tissue by filtering through a polyester gauze mesh with 100 µm apertures. The cell suspension was centrifuged at 300rpm for 5 minutes and the pellet washed again with a large volume of medium. Briefly, 20 µl of the cell suspension was added to 20 µl of Trypan Blue dye in a sterile capped tube and inverted briefly to mix. This mixture was introduced, into the chamber of an Improved Neubauer haemocytometer and the chamber was examined by light microscopy at 100x magnification. Total hepatocyte numbers were counted in the entire 1 mm delineated field of the haemocytometer and seeded in the desired concentration on collagen-coated plastic ware.

5.9 Manipulation of murine cells

5.9.1 Production of lentivirus

Lentiviral vector production was performed by transient transfection of plasmids PLP1, PLP2 and VSV-G encoding helper functions along with the expression cassette in 293T cells as described previously [106].

5.9.2 Conditional immortalization of MEFs

With subsequent sub culturing, primary cells lose replicative potential and proceed towards Hayflick's limit; a point after which cells undergo senescence and die. Immortalised cells

often replace primary cells in routine studies as these cells expressing certain oncogenes continue to proliferate even after repeated passaging. Owing to the disadvantages of maintaining primary cell cultures, and the plausible changes induced by induction of the oncogenes a strategy called 'Conditional immortalization' was developed. Conditionally immortalized cells contain oncogenes that are activated upon induction but retain 'primary like' phenotype otherwise. To generate immortalized MEFs, 2 wells of a 6-well dish were plated with the desired cell line and one was transduced with lentivirus coding for SV40 large T antigen while the other well was left untransduced. The transduced cell line was termed 'immortalized' when upon repeated sub-culturing the primary cells had lost replication potential and ceased to grow as compared to the transduced cell line which in the induced state continued to proliferate.

5.9.3 Lentiviral transduction of miR122 in MEFs

2x10*5 MEF cells were seeded on a 12-well plate, one day after which the media was aspirated and 500ul of lentivirus stock solution supplemented with 4mg/ml Polybrene was added to the cells. After over-night incubation, the cell monolayer was washed once with 1x PBS and replenished with fresh media.

5.10 Culture and manipulation of *E.coli*

5.10.1 *E.coli* laboratory strains

Table 3: List of Bacterial strains -

E. coli strain	***Genotype***
DH10B	*F', mcrA, (mrr hsdRMS-mcrBC), Φ80dlacZ M15, LacX74, deoR, recA1, ara139, galU, galK, λ - , rpsL, endA1, nupG (Gibco BRL)*
TOP10	*F- mrcA Δ (mrr-hsdRMS-mcrBC) Φ80dlacZM15, ΔlacX74 recA1 araD139 Δ (ara-leu) 7697 galU galK rpsL (StrR) endA1 nupG*
INV110	*F ´ (traΔ36 proAB lacIq lacZΔM15) rpsL (StrR) thr leu endA thi-1 lacY galK alT ara tonA tsx dam dcm supE44 Δ(lac-proAB) Δ(mcrC-mrr)102::Tn10 (TetR)*

5.10.2 Culture media and antibiotics

Table 4: List of Culture media -

Bacterial Medium	Composition
Lysogeny Broth (LB)	1 % (w/v) tryptone, 0.7 % (w/v) NaCl, 0.5 % (w/v) yeast extract, adjusted to pH 7.5 with NaOH
LB-Agar	LB, 1.5 % (w/v) agar-agar
Ampicillin	Stock solution: 5 mg/ml in ethanol, filtered sterile 5 µg/ml in LB-medium 25 mg/ml in agar culture plates
Kanamycin	Stock solution: 5 mg/ml in double distilled water, filtered sterile 2.5 µg/ml in LB-medium 12.5 µg/ml in agar culture plates

Agar culture plates

15 g of solid agar in 1 L of LB-medium was autoclaved under conditions mentioned above and poured into plates with the required antibiotic.

5.10.3 Preparation of chemocompetent *E.coli* strain

Cells were grown at 37°C, 180 rpm until the cell suspension had reached an OD 600 between 0.6 – 0.8. Subsequently, cells were centrifuged for 5 - 10 min in a cooled centrifuge at 1,500 rpm. The cell pellet was resuspended in cold sterile water, washed twice with cold sterile water and once with 10% glycerol. Finally, bacterial cell pellet was resuspended in appropriate amounts of 10% glycerol and stored immediately at -80° C.

5.10.4 Chemical transformation of DNA into *E.coli*

TOP10 cells frozen at -80°C were thawed gradually on ice. 50ng of DNA was added to the thawed cells and heat shocked at 37°C for 10 minutes. The cells were placed on ice for 5 minutes after which the cell suspension was incubated at 37°C in the presence of 1ml LB media. Subsequently, the suspension was spread on to agar culture plates containing the antibiotic corresponding to that carried as a resistance gene in the plasmid.

5.10.5 Preservation of bacterial strains

For short-term storage, *E. coli* was cultured over-night at 37°C on agar plates and subsequently kept at 4°C. For long-term storage, the bacterial suspension was

mixed with 87% glycerol solution in a ratio of 1:1 and preserved in glass vials at -20°C or -80°C.

5.11 Culture and manipulation of eukaryotic cells

5.11.1 Cell lines

Table 5: List of Cell lines -

Cell line	Descrption
1˚ MEFs	Primary Mouse embryonic fibroblasts
Conditionally immortalised MEFs	Murine Fibroblasts immortalised with SV40 large T antigen
1˚m.hepatocyte	Primary murine hepatocytes

5.11.2 Culture media and reagents

Table 6: List of Culture media and Reagents –

Medium	Composition
Dulbecco's Modified English Media (DMEM)	13.63 g/L DMEM powder (Sigma), 3.67 g/L (44mM) $NaHCO_3$, 2.6g/L 10mM HEPES, pH 7.2
Phosphate buffered Saline (PBS)	140 mM NaCl, 27 mM KCl, 7.2 mM Na_2HPO_4 , 14.7mM KH_2PO_4 , pH 6.8-7.0
Trypsin EDTA PBS (TEP)	6mM EDTA, 0.1% trypsin (Gibco) in PBS
100 x Pen/Strep	6.06 mg/ml ampicillin (10,000 U/ml), 10mg/ml streptomycin, pH to 7.4 with NaOH and stored at -20°C
100x Non essential amino acids	Supplied by Gibco
Fetal Calf Serum (FCS)	Supplied by PAA
G418	100mg/ml G418 in water, filtered sterile, (stored at -20°C)
Puromycin	5 mg/ml in water, filtered sterile, (stored at -20°C)

<u>DMEM 3+E for hepatoma cells:</u>

DMEM, 1 x Pen/Strep, 1 x Glutamine, 10% FBS, 1x Non essential amino acids

<u>Intestinal Epithelium Media:</u>

DMEM/HAM F12 (1:1), 1 % Glu, 1 % PenStrep, 2 % FCS, 20 mM HEPES, 5 µg/ml Insulin (Sigma), 5 µg/ml humanes Apo-Transferrin (Sigma), 50 nM Dexamethasone

Media for MEFs:

DMEM, 1 x Pen/Strep, 1 x Glutamine, 10% FBS

5.11.3 Cell cultivation

Cells were maintained in DMEM media supplemented with 10% Penicillin and Streptomycin, 10% L-Glutamate, 10% Non-essential amino acids in addition to 10% Fetal bovine serum. These cells were maintained in an incubator at 37˚C and 5% CO_2 and maximal relative humidity. Cells were sub-cultured every 3-4 days. To sub-culture, cell monolayers were washed with PBS and then Trypsinized for a few minutes at 37˚C until the cells dislodged from the primary culture vessel. The resultant cell suspension was counted and seeded further in the desired concentration.

5.11.4 Estimation of cell density

Cell suspension to be analyzed was diluted in Trypan blue (1:10). 10µl of the mixture was added to the chamber by gently resting the end of the tip at the edge of the chambers. Cells in 5 square segments were counted and the average was used to determine the total cell count. The formula used was as follows:

Cells density (per ml) = X (average cell count)*5*(multiplication factor)*10(dilution factor)

Multiplication factors: 1000- Neubauer Chamber or 5000- Fuchs-Rosenthal chamber

5.11.5 Long term preservation of cells

Cryopreservation is performed to preserve cells for long term storage and is carried out by gradual cooling in the presence of a cryoprotective agent like Dimethylsulfoxide. Gradual freezing reduces the risk of ice crystal formation and cell damage. To this end, trypsinized cell suspensions were centrifuged at 1000rpm for 5 minutes and the cell pellet obtained was resuspended in 10% DMSO diluted in cold FBS. The mix was then transferred into cryovials and stored in an ice box for an hour, after which, they were transferred to a -20˚C freezer for 2 days. Following this, the vials were then transferred to a liquid Nitrogen chamber for long time preservation.

In order to thaw cells preserved in a liquid Nitrogen chamber, the cryovials were thawed immediately by plunging vials into a 37˚C water bath. The vial was then washed with Ethanol and the cell suspension was resuspended in optimal media. The suspension was then centrifuged at 1000rpm for 5 minutes and the cell pellet thus obtained was added to fresh media and seeded on appropriate cell culture dishes.

5.12 Nucleic acid transfection into cells

5.12.1 Plasmid DNA transfection

Lipofection was carried out on Huh7 and Huh7.5 cells that were seeded at a concentration of 5x10*5 on 12-well plates and transfected as per guided protocol provided in the Lipofectamine2000 kit by Invitrogen.

5.12.2 RNA transfection by Lipofection

Lipofection was carried out on Huh7, Huh7.5 and primary hepatocytes that were seeded at a concentration of 5x10*5 on 12-well plates and 3µg RNA was transfected per well according to the protocol provided in the Lipofectamine2000 kit.

5.12.3 RNA transfection by electroporation

1x10*7 cells (Huh7 cells and fibroblasts) and 1.5x10*7 cells (Huh7.5 cells) were mixed with 2mM ATP and 5mM L-glutathione at a final volume of 400µl of cytomix (120 mM KCl; 0.15 mM $CaCl_2$; 10 mM K_2HPO_4/KH2PO_4 (pH 7.6); 25 mM Hepes (Gibco, cell culture grade); 2 mM EGTA; 5 mM $MgCl_2$; adjust pH to 7.6 with KOH, filter strilised). The desired concentration of RNA was added and mixed thoroughly. This suspension was placed in an electroporation cuvette (0.4cm) and electroporated at 270V and 975µF. In order to inhibit secreted IFN neutralizing antibodies against type I IFN was added to the freshly electroporated cells. Electroporation efficiency was measure 4 hours post electroporation.

5.13 Isolation and preparation of nucleic acids

5.13.1 RNA isolation from cells

RNA was isolated from eukaryotic cells with the RNeasy Mini Kit (Qiagen) according to manufacturer's instruction. This technology is based on the selective binding properties of a silica-gel-based membrane. A specialized high-salt buffer system allows up to 100 µg of RNA longer than 200 bases to bind to the RNeasy silica-gel membrane. Therefore, cultured cells (~1 *10^7 cells) were first harvested and homogenized in the presence of 600 µl of a highly denaturating guanidine isothiocyanate-containing buffer, which immediately inactivates RNases to ensure isolation of intact RNA. Then, 600 µl of ethanol were added to provide appropriate binding conditions. 700 µl of this solution were applied to an RNeasy mini column where the total RNA bound to the membrane and contaminants were washed away. Afterwards, RNA was eluted in 50 µl of RNase-free water.

5.13.2 Small scale isolation of plasmid DNA

Table 7: List of buffers for Plasmid DNA isolation -

Materials	Descrption
STET buffer	80 g/L Sucrose, 0.5% Triton X100, 50mM EDTA, 10 mM Tris/HCl pH8.0
TE buffer	0.1 mM EDTA, 10 mM Tris/HCL, pH 8.0
Lysozyme	10 mg/ml Lysozyme in TE buffer
Ammonium acetate	8 M NH_4OAc
TE with RNase	10 µg/ml RNase A in TE buffer

2 ml of LB-medium containing the required antibiotic were inoculated with the respective E.coli clone and cultured over-night at 37°C, 180 rpm. Bacterial cell suspensions were transferred to 2 ml reaction tubes, centrifuged (6,000 rpm, 2 min) and the supernatant was discarded. Pelleted cells were then re-suspended in 500 µl STET buffer by thorough vortexing. After addition of 50 µl lysozyme solution, samples were incubated for 2 - 3 min at room temperature for subsequent digestion of the cell walls. The reaction was stopped and DNA denatured by heat (95°C, 90 s), subsequent centrifugation (13,000 rpm, 5 min) separates genomic DNA that does not renature. The viscous pellets resulting from digested

proteins were removed with toothpicks and 50 µl ammonium acetate and 500 µl isopropanol were added to the lysate. After centrifugation (13,000 rpm, 5 min) and removal of supernatant, the DNA pellet was washed with 500 µl 70% ethanol. Pellets were dried thoroughly at 37°C. When transparent, pellets were dissolved in TE + RNase (50 µl).

5.13.3 Large scale isolation of plasmid DNA

For large-scale isolation of plasmid DNA, the Plasmid Maxi Kit (Qiagen) or PureYield Plasmid Midiprep (Promega) kits were used according to the manufacturer's instructions. Both technologies base on alkaline lysis of bacteria and purification of DNA via silica membranes. 200 – 500 ml bacterial suspension grown in LB-medium and appropriate antibiotics at 37°C, 180 rpm over-night were used. DNA was dissolved in appropriate amounts of TE buffer and authenticity checked by agarose gel electrophoresis and quantified using a Nanodrop-2000.

5.13.4 Agarose gel electrophoresis

Table 8: List of buffers for Agarose gel electrophoresis -

1x TAE buffer:	40mM Tris acetate (pH 7.5) 20mM Sodium acetate 1mM EDTA
5x loading buffer:	15% Ficoll, 50mM EDTA, 1X TAE, 0.05% Bromophenol blue, 0.05% Xylenecyanol

1% agarose gel was made by adding 1gram of agarose to 100ml 1X TAE and bringing to a boil in a microwave oven. Once slightly cooled, 2 µl Ethidium bromide or 1.5 µlMidori Green was added and the gel was poured into a gel chamber with an appropriate comb in place. The set gel was then placed in an electrophoresis tank in 1x TAE buffer. Prior to loading, the DNA samples were mixed with 5x loading buffer. A DNA ladder was loaded on the gel to determine the size of the DNA

5.13.5 Purification of DNA from gels

DNA bands excised from the agarose gels were purified using the QiaQuick gel extraction kit from Qiagen according to manufacturer's instructions.

5.13.6 Purification of DNA from PCR samples

PCR samples were mixed with 3 times PB buffer and then eluted through a column using PE buffer as per vendor's (QiaQuick gel extraction kit from Qiagen) instructions.

5.13.7 Sequencing

DNA sequencing was performed by the gene sequencing platform provided by the Gene Analysis Department, Helmholtz Centre for Infection Research, Braunschweig.

5.14 DNA modifications

5.14.1 Restriction analysis of DNA

Restriction of plasmids and PCR products was performed according to manufacturer's (NEB) instructions on the basis of in-silico digestion on Vector NTI software.

5.14.2 5' overhang fill ups

DNA was dissolved in 1X NEBuffer 1 or T4 DNA Ligase Reaction Buffer supplemented with 33 μM each dNTP. 1 unit Klenow per microgram DNA was incubated for 15 minutes at 25°C. The reaction was stopped by heating at 75°C for 20 minutes.

5.14.3 Site directed Mutagenesis:

The primers were designed in accordance with the primer design guidelines available at the Stratagene website (http://www.genomics.agilent.com/CollectionSubpage.aspx?PageType=Tool&SubPageType=ToolQCPD&PageID=15) and the reactions were performed as per instructions.

5.14.4 Dephophorylation of DNA fragments

10 x Phosphatase buffer	500 mM Tris/HCl (pH 9.0), 10 mM MgCl 2 , 1 mM ZnCl 2 , 1 mM Spermidine

To prevent religation of a restricted vector, 5' and 3' overhanging ends were dephosphorylated by alkaline phosphatase (shrimp alkaline phosphatase (SAP) or calf intestine phosphatase (CIP)). DNA with overhanging ends was incubated with 1 x phosphatase buffer and SAP / CIP at 37°C for 30min. The reaction was stopped by heat inactivation (20 min at 80°C).

5.14.5 Ligation of DNA fragments

5 x Ligase buffer	250 mM Tris/HCl (pH7.6), 50 mM MgCl 2 , 25% (w/v) PEG8000, 5 mM ATP, 5 mM DTT

Ligation of plasmids backbone and insert was performed according to manufacturer's (NEB) instructions on the basis of in-silico digestion on Vector NTI software. A ratio of 1:10 plasmid: insert was incubated with 1x ligase buffer and 2U T4-DNA Ligase (NEB) in a total volume of 10 µl for 3 hours or longer at room temperature. 2 µl of the resulting ligation mixture was used for transformation.

5.14.6 Hybridization of oligonucleotides

Annealing buffer	100mM Potassium acetate, 30mM HEPES-KOH (pH 7.4), 2mM Magnesium acetate

In order to anneal oligonucleotides, 100pmol/µl of each oligonucleotide was added to 48µl of annealing buffer and incubated at 95˚C for 4 minutes. Following this, the reaction was incubated at 70˚C for 10 minutes after which it was brought to room temperature and stored at 4˚C until further use.

5.14.7 Phosphorylation of oligonucleotides

In order to phosphorylate oligonucleotides, 2µl of the desired reaction was incubated in the presence of 1µl of T4 Polynucleotide Kinase buffer, 1mM ATP, 1µl of T4 Polynucleotide Kinase and 5 µl of double distilled water at 37˚C for 30 minutes followed by a 10 minute incubation period at 70˚C.

5.15 Analysis of nucleic acids

PCR

PCR was carried out for amplifying specific regions of plasmid DNA. Either

i. BioMix 2x ready mix (Bioline) or
ii. Expand Long Template PCR system (Roche)

possessing inherent 3'-5' exonuclease or proof-reading activity was used. PCR comprises a sequence of 3 basic steps that are repeated in 25 - 35 cycles:

Denaturation: dsDNA is melted to single strands by high temperatures (94 -96°C)

Annealing: annealing requires lower temperatures for binding of the primers to their complementary sequences (temperature is chosen 5 – 10°C below calculated melting temperature)

Extension: involves synthesis of complementary strands using the polymerase enzyme.

PCR using Expand Long Template System

Materials	**Volume**
Supplied PCR buffer (10 x conc)	5µl
10 (pmol/µl) forward primer	1 µl
10 (pmol/µl) reverse primer	1 µl
DMSO	1 – 2.5 µl
DNA polymerase mix (Roche) (3.75 U)	0.75 µl
DNA template	as required
Water	upto 50µl

PCR program using the T3 thermocycler (Biometra)

Steps	Temperature	Time
1	95	2 min
2	95	10 sec
3	58-62	30 sec
4	72	1 min / kb of product
5	72	7 min
6	16	pause

steps 2 – 4 repeated 25 - 35 times

PCR using BioMix (Bioline) (50 µl total)

Materials	Volume
2 x BioMix	5µl
10 (pmol/µl) forward primer	1 µl
10 (pmol/µl) reverse primer	1 µl
DMSO	2.5 µl
DNA polymerase mix (Roche) (3.75 U)	0.75 µl
DNA template	as required (0.1 – 50 ng)
Water	up to 50µl

PCR program using the T3 thermocycler (Biometra)

Steps	Temperature	Time
1	95	2 min
2	95	10 sec
3	58-62	30 sec
4	72	45 sec / kb of product
5	72	7 min
6	16	pause

steps 2 – 4 repeated 25 - 35 times

Preparative PCR was carried out in 50 µl, analytical PCR in 20 µl of total volume.

5.16 Quantitative real time PCR

Total cellular RNA was isolated as described above. RNA in a concentration of 100ng/µl was added to cDNA ready-to-go first prime tube (GE health care) in the presence of oligo dT (0.5µg/3µL) and incubated at 37°C for 1 hour. The resulting cDNA mixture was diluted 10 times and RT-PCR was carried out to determine induction of IFN stimulated genes relative to a house keeping gene Beta-actin.

5.17 NS5B polymerase inhibition assay

In order to verify if the polymerase competent strain (JFH-1-prom3) could initiate and maintain replication after Tet induced transcription, a replication assay in the presence of NS5B polymerase inhibitor 2-CMA was performed. To this end, Huh7.5 cells transiently transfected with JFH-1-prom3 (POL+) or JFH-1ΔGDD-prom3 was co-transfected with lenti tTA and a transfection control vector expressing Renilla luciferase (MDICRluc). Upon transfection, the cells were maintained in the absence of Doxycycline for three days in order to aid Tet dependent transcription. Following this, the cells were cultured in media supplemented with Doxycycline (2µg/ml) in the presence or absence of 3.5µM of NS5B polymerase inhibitor 2-CMA. Luciferase values were measured after 29 hours and 39 hours of incubation.

5.18 Luciferase assays

Luciferase buffer	25 mM glycylglycine, 15 mM $MgSO_4$ in H_2O, pH 7.8 (stored at 4°C)
ATP solution	5 mM ATP in H_2O, pH 7.5 (stored at -20°C)
Luciferin solution	0.1 mM synthetic D-luciferin (Promega), 25 mM glycylglycine in water, pH 7.8 (stored at -20°C)
Reaction buffer	ATP solution: luciferase buffer (1:5)

Cells were washed with PBS and incubated with appropriate amounts of passive lysis buffer (PLB, Promega) at room temperature for 15 min, shaking. For luminometric assay 10 µl cell lysate was added to 400 µl reaction buffer and 50 µl of luciferin were injected at the luminometer, respectively. As firefly luciferase converts

the substrate luciferin in the presence of ATP and oxygen, light is emitted and can be measured at 610 nm in a luminometer. Alternatively, lucifersse assays were also performed using Firefly luceiferase from Promega. To this end, 20 µl lysate was added to 100 µl luciferin and the read out was measured at 610 nm.

5.19 Type 1 Interferon detection

5.19.1 VSV inhibition bioassay

LMTK$^-$ cells were incubated for 2 hours in the presence of supernatant aspirated from electroporated MEFs. Subsequently, cells were infected with VSV-GFP (1.45x10*4 pfu) for 1 hour and then incubated in DMEM3+ media for 24 hours. Decrease in GFP expression was measured using flow cytometry and was indicative of the antiviral activity in the supernatants.

5.19.2 Mx2 reporter IEC

Intestinal epithelial cells isolated from a reporter mouse expressing luciferase under the control of an Mx2 promoter were incubated along with the supernatant aspirated from electroporated MEFs. Induction of Mx2 upon IFN stimulation from the supernatant was measured as luciferase response.

5.20 Flow cytometry

Antibodies

Table 9: List of Antibodies -

	Name	Source
1	Anti-NS3 (HCV)	Prof.Thomas Pietschmann, TWINCORE
2	Anti-NS5A (HCV)	Prof.Thomas Pietschmann, TWINCORE
3	Human NGFR(CD 271)	Biolegend
4	Anti IFN-α (neutralizing)	(4EA1) Dr.Lienenklaus, HZI, Braunschweig
5	Anti IFN-β (neutralizing)	(7FD3) Dr.Lienenklaus, HZI, Braunschweig

Flow cytometry and cell sorting

Hepatoma cells stably transfected with the inducible HCV construct along with the lentiviral plasmid coding for the transactivator or reverse transactivator were detached from plates using trypsin and washed with PBS, stained with antibody or fixed and permeabilized with BD Cytofix/Cytoperm Kit (BD Biosciences). Cells were stained for an hour with antibodies against human NGFR (Biolegend) at 20µg/ml conjugated to PE or APC washed twice and re-suspended in FACS buffer. Cell fluorescence was analyzed using an LSRII (BD Biosciences) flow cytometer and FlowJo FACS analysis program (TreeStar, Ashland, OR).MEFs were detached from plates using trypsin after one wash with PBS, the cells were centrifuged and washed using 1x PBS. The pellet was resuspended in appropriate volume of FACS buffer. Cells were sorted for GFP positive miR-122 cells on the BD FACS Aria. The sorted cells were subsequently cultured in the presence of gentamicin sulfate at a final concentration of 1:200 to avoid plausible bacterial contamination.

5.21 Stable cell lines

Huh7.5 and Huh7 cells stably expressing the replication proficient and deficient HCV subgenoimc replicon driven by the tet promoter or the newer pTight tet promoter in the presence of a transactivator or reverse transactivator were generated by selecting for

blasticidin resistant clones. All transfection were carried out using Lipofectamine 2000 following manufacturer's instructions along with the co-transfection of a plasmid coding for blasticidin resistance. Cells were selected with 7.5µg/ml Blasticidin. The generated clones were tested for expression and induction.

5.22 Indirect immunoflourescence

For immunoflourescence, cells were plated at 2x10^5/ml density on 24 well BD Falcon CultureSlides (BD Biosciences, San Jose, CA) and grown overnight at 37°C, 5% CO2. Cells were washed with PBS and fixed with 4% paraformaldehyde in a 1:1 ratio for 10 minutes. Slides were dried, rinsed with PBS, blocked for 1 hour with 1%Bovine serum albumin in PBS in a 1:1 ratio and probed with anti-HCV NS3 antibody in a 1:200 ratio of dilution overnight at 4°C. Slides were washed with 0.5% Tween 20-PBS for 30 min followed by staining for FITC goat anti-mouse IgG antibody 1:1000 (Invitrogen) for 1 hour. During the final wash step DAPI (2 µg/ml) was added for 10 minutes. Coverslips were mounted on a glass slide over a drop of Mowiol and analyzed for fluorescence using a Leica microscope.

1 Li, X. D., Sun, L., Seth, R. B., Pineda, G. & Chen, Z. J. Hepatitis C virus protease NS3/4A cleaves mitochondrial antiviral signaling protein off the mitochondria to evade innate immunity. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 17717-17722, doi:10.1073/pnas.0508531102 (2005).

2 Li, K. *et al.* Immune evasion by hepatitis C virus NS3/4A protease-mediated cleavage of the Toll-like receptor 3 adaptor protein TRIF. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 2992-2997, doi:10.1073/pnas.0408824102 (2005).

3 Kumthip, K. *et al.* Hepatitis C Virus NS5A Disrupts STAT1 Phosphorylation and Suppresses Type I Interferon Signaling. *Journal of virology* **86**, 8581-8591, doi:10.1128/JVI.00533-12 (2012).

4 Stojdl, D. F. *et al.* VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* **4**, 263-275 (2003).

5 Stirnweiss, A. *et al.* IFN regulatory factor-1 bypasses IFN-mediated antiviral effects through viperin gene induction. *Journal of immunology* **184**, 5179-5185, doi:10.4049/jimmunol.0902264 (2010).

6 Feinstone, S. M., Kapikian, A. Z., Purcell, R. H., Alter, H. J. & Holland, P. V. Transfusion-associated hepatitis not due to viral hepatitis type A or B. *The New England journal of medicine* **292**, 767-770, doi:10.1056/NEJM197504102921502 (1975).

7 Seeff, L. B. *et al.* Long-term mortality after transfusion-associated non-A, non-B hepatitis. The National Heart, Lung, and Blood Institute Study Group. *The New England journal of medicine* **327**, 1906-1911, doi:10.1056/NEJM199212313272703 (1992).

8 Choo, Q. L. *et al.* Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* **244**, 359-362 (1989).

9 Simmonds, P. *et al.* Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *The Journal of general virology* **74 (Pt 11)**, 2391-2399 (1993).

10 Johnson, D. C. & Huber, M. T. Directed egress of animal viruses promotes cell-to-cell spread. *Journal of virology* **76**, 1-8 (2002).

11 2002, W. H. O.

12 Pawlotsky, J. M. Therapy of hepatitis C: from empiricism to eradication. *Hepatology* **43**, S207-220, doi:10.1002/hep.21064 (2006).

13 Lavanchy, D. The global burden of hepatitis C. *Liver Int* **29 Suppl 1**, 74-81, doi:10.1111/j.1478-3231.2008.01934.x (2009).

14 Shepard, C. W., Finelli, L. & Alter, M. J. Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* **5**, 558-567, doi:10.1016/S1473-3099(05)70216-4 (2005).

15 Hwang, L. Y. *et al.* Relationship of cosmetic procedures and drug use to hepatitis C and hepatitis B virus infections in a low-risk population. *Hepatology* **44**, 341-351, doi:10.1002/hep.21252 (2006).

16 De Carli, G., Puro, V., Ippolito, G. & Studio Italiano Rischio Occupazionale da, H. I. V. G. Risk of hepatitis C virus transmission following percutaneous exposure in healthcare workers. *Infection* **31 Suppl 2**, 22-27 (2003).

17 Seeff, L. B. Natural history of chronic hepatitis C. *Hepatology* **36**, S35-46, doi:10.1053/jhep.2002.36806 (2002).

18 Seeff, L. B. The natural history of chronic hepatitis C virus infection. *Clin Liver Dis* **1**, 587-602 (1997).

19 Puro, V. *et al.* Occupational hepatitis C virus infection in Italian health care workers. Italian Study Group on Occupational Risk of Bloodborne Infections. *Am J Public Health* **85**, 1272-1275 (1995).

20 Koretz, R. L. *et al.* Non-A, non-B posttransfusion hepatitis: comparing C and non-C hepatitis. *Hepatology* **17**, 361-365 (1993).

21 Seeff, L. B. Hepatitis C from a needlestick injury. *Ann Intern Med* **115**, 411 (1991).

22 Hoofnagle, J. H. Course and outcome of hepatitis C. *Hepatology* **36**, S21-29, doi:10.1053/jhep.2002.36227 (2002).

23 Aach, R. D. *et al.* Hepatitis C virus infection in post-transfusion hepatitis. An analysis with first- and second-generation assays. *The New England journal of medicine* **325**, 1325-1329, doi:10.1056/NEJM199111073251901 (1991).

24 Koretz, R. L., Abbey, H., Coleman, E. & Gitnick, G. Non-A, non-B post-transfusion hepatitis. Looking back in the second decade. *Ann Intern Med* **119**, 110-115 (1993).

25 Jaeckel, E. *et al.* Treatment of acute hepatitis C with interferon alfa-2b. *The New England journal of medicine* **345**, 1452-1457, doi:10.1056/NEJMoa011232 (2001).

26 Gerlach, J. T. *et al.* Acute hepatitis C: high rate of both spontaneous and treatment-induced viral clearance. *Gastroenterology* **125**, 80-88 (2003).

27 Manns, M. P. *et al.* Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* **358**, 958-965 (2001).

28 Fried, M. W. *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *The New England journal of medicine* **347**, 975-982, doi:10.1056/NEJMoa020047 (2002).

29 Hadziyannis, S. J. *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* **140**, 346-355 (2004).

30 Neumann, A. U. *et al.* Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* **282**, 103-107 (1998).

31 Sarrazin, C. & Zeuzem, S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* **138**, 447-462, doi:10.1053/j.gastro.2009.11.055 (2010).

32 Houghton, M. & Abrignani, S. Prospects for a vaccine against the hepatitis C virus. *Nature* **436**, 961-966, doi:10.1038/nature04081 (2005).

33 Dustin, L. B. & Rice, C. M. Flying under the radar: the immunobiology of hepatitis C. *Annu Rev Immunol* **25**, 71-99, doi:10.1146/annurev.immunol.25.022106.141602 (2007).

34 Meunier, J. C. *et al.* Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 4560-4565, doi:10.1073/pnas.0501275102 (2005).

35 Fletcher, N. F. *et al.* Hepatitis C virus infects the endothelial cells of the blood-brain barrier. *Gastroenterology* **142**, 634-643 e636, doi:10.1053/j.gastro.2011.11.028 (2012).

36 Kondo, Y., Sung, V. M., Machida, K., Liu, M. & Lai, M. M. Hepatitis C virus infects T cells and affects interferon-gamma signaling in T cell lines. *Virology* **361**, 161-173, doi:10.1016/j.virol.2006.11.009 (2007).

37 Inokuchi, M. *et al.* Infection of B cells with hepatitis C virus for the development of lymphoproliferative disorders in patients with chronic hepatitis C. *J Med Virol* **81**, 619-627, doi:10.1002/jmv.21388 (2009).

38 Kato, T. *et al.* Nonhepatic cell lines HeLa and 293 support efficient replication of the hepatitis C virus genotype 2a subgenomic replicon. *Journal of virology* **79**, 592-596, doi:10.1128/JVI.79.1.592-596.2005 (2005).

39 Chang, J. *et al.* Liver-specific microRNA miR-122 enhances the replication of hepatitis C virus in nonhepatic cells. *Journal of virology* **82**, 8215-8223, doi:10.1128/JVI.02575-07 (2008).

40 Lin, L. T. *et al.* Replication of subgenomic hepatitis C virus replicons in mouse fibroblasts is facilitated by deletion of interferon regulatory factor 3 and expression of liver-specific microRNA 122. *Journal of virology* **84**, 9170-9180, doi:10.1128/JVI.00559-10 (2010).

41 Ploss, A. *et al.* Human occludin is a hepatitis C virus entry factor required for infection of mouse cells. *Nature* **457**, 882-886, doi:10.1038/nature07684 (2009).

42 Zeisel, M. B. *et al.* Scavenger receptor class B type I is a key host factor for hepatitis C virus infection required for an entry step closely linked to CD81. *Hepatology* **46**, 1722-1731, doi:10.1002/hep.21994 (2007).

43 Liu, S. *et al.* Tight junction proteins claudin-1 and occludin control hepatitis C virus entry and are downregulated during infection to prevent superinfection. *Journal of virology* **83**, 2011-2014, doi:10.1128/JVI.01888-08 (2009).

44 Pileri, P. *et al.* Binding of hepatitis C virus to CD81. *Science* **282**, 938-941 (1998).

45 Agnello, V., Abel, G., Elfahal, M., Knight, G. B. & Zhang, Q. X. Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. *Proceedings of the National Academy of Sciences of the United States of America* **96**, 12766-12771 (1999).

46 Molina, S. *et al.* The low-density lipoprotein receptor plays a role in the infection of primary human hepatocytes by hepatitis C virus. *J Hepatol* **46**, 411-419, doi:10.1016/j.jhep.2006.09.024 (2007).

47 Wang, C., Sarnow, P. & Siddiqui, A. Translation of human hepatitis C virus RNA in cultured cells is mediated by an internal ribosome-binding mechanism. *Journal of virology* **67**, 3338-3344 (1993).

48 Pereira, A. A. & Jacobson, I. M. New and experimental therapies for HCV. *Nat Rev Gastroenterol Hepatol* **6**, 403-411, doi:10.1038/nrgastro.2009.92 (2009).

49 Gosert, R. *et al.* Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. *Journal of virology* **77**, 5487-5492 (2003).

50 Schmidt, A. *et al.* 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 12067-12072, doi:10.1073/pnas.0900971106 (2009).

51 Rehwinkel, J. *et al.* RIG-I detects viral genomic RNA during negative-strand RNA virus infection. *Cell* **140**, 397-408, doi:10.1016/j.cell.2010.01.020 (2010).

52 Kato, H. *et al.* Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* **205**, 1601-1610, doi:10.1084/jem.20080091 (2008).

53 Kato, H. *et al.* Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* **441**, 101-105, doi:10.1038/nature04734 (2006).

54 Bowie, A. & O'Neill, L. A. The interleukin-1 receptor/Toll-like receptor superfamily: signal generators for pro-inflammatory interleukins and microbial products. *Journal of leukocyte biology* **67**, 508-514 (2000).

55 Akira, S., Uematsu, S. & Takeuchi, O. Pathogen recognition and innate immunity. *Cell* **124**, 783-801, doi:10.1016/j.cell.2006.02.015 (2006).

56 Krug, A. *et al.* Herpes simplex virus type 1 activates murine natural interferon-producing cells through toll-like receptor 9. *Blood* **103**, 1433-1437, doi:10.1182/blood-2003-08-2674 (2004).

57 Panne, D., Maniatis, T. & Harrison, S. C. Crystal structure of ATF-2/c-Jun and IRF-3 bound to the interferon-beta enhancer. *The EMBO journal* **23**, 4384-4393, doi:10.1038/sj.emboj.7600453 (2004).

58 Yoneyama, M. *et al.* Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *The EMBO journal* **17**, 1087-1095, doi:10.1093/emboj/17.4.1087 (1998).

59 Lin, R., Heylbroeck, C., Pitha, P. M. & Hiscott, J. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation. *Mol Cell Biol* **18**, 2986-2996 (1998).

60 Sato, M. *et al.* Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett* **441**, 106-110 (1998).

61 Sato, M. *et al.* Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN-alpha/beta gene induction. *Immunity* **13**, 539-548 (2000).

62 Hiscott, J. Triggering the innate antiviral response through IRF-3 activation. *The Journal of biological chemistry* **282**, 15325-15329, doi:10.1074/jbc.R700002200 (2007).

63 Gale, M., Jr. & Foy, E. M. Evasion of intracellular host defence by hepatitis C virus. *Nature* **436**, 939-945, doi:10.1038/nature04078 (2005).

64 Rani, M. R. & Ransohoff, R. M. Alternative and accessory pathways in the regulation of IFN-beta-mediated gene expression. *J Interferon Cytokine Res* **25**, 788-798, doi:10.1089/jir.2005.25.788 (2005).

65 Bigger, C. B., Brasky, K. M. & Lanford, R. E. DNA microarray analysis of chimpanzee liver during acute resolving hepatitis C virus infection. *Journal of virology* **75**, 7059-7066, doi:10.1128/JVI.75.15.7059-7066.2001 (2001).

66 Sarasin-Filipowicz, M. *et al.* Interferon signaling and treatment outcome in chronic hepatitis C. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 7034-7039, doi:10.1073/pnas.0707882105 (2008).

67 Hershey, J. W. Translational control in mammalian cells. *Annu Rev Biochem* **60**, 717-755, doi:10.1146/annurev.bi.60.070191.003441 (1991).

68 Townsend, H. L. *et al.* A viral RNA competitively inhibits the antiviral endoribonuclease domain of RNase L. *RNA* **14**, 1026-1036, doi:10.1261/rna.958908 (2008).

69 Malathi, K., Dong, B., Gale, M., Jr. & Silverman, R. H. Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature* **448**, 816-819, doi:10.1038/nature06042 (2007).

70 Park, H. *et al.* IL-29 is the dominant type III interferon produced by hepatocytes during acute hepatitis C virus infection. *Hepatology*, doi:10.1002/hep.25897 (2012).

71 Kato, T., Esumi, M., Yamashita, S., Abe, K. & Shikata, T. Interferon-inducible gene expression in chimpanzee liver infected with hepatitis C virus. *Virology* **190**, 856-860 (1992).

72 Burgio, V. L. *et al.* Expression of co-stimulatory molecules by Kupffer cells in chronic hepatitis of hepatitis C virus etiology. *Hepatology* **27**, 1600-1606, doi:10.1002/hep.510270620 (1998).

73 Hosomura, N. *et al.* HCV-related proteins activate Kupffer cells isolated from human liver tissues. *Dig Dis Sci* **56**, 1057-1064, doi:10.1007/s10620-010-1395-y (2011).

74 Jin, Y. *et al.* The immune reactivity role of HCV-induced liver infiltrating lymphocytes in hepatocellular damage. *J Clin Immunol* **17**, 140-153 (1997).

75 Tseng, C. T., Miskovsky, E., Houghton, M. & Klimpel, G. R. Characterization of liver T-cell receptor gammadelta T cells obtained from individuals chronically infected with hepatitis C virus (HCV): evidence for these T cells playing a role in the liver pathology associated with HCV infections. *Hepatology* **33**, 1312-1320, doi:10.1053/jhep.2001.24269 (2001).

76 Shen, T. *et al.* Characteristics and PD-1 expression of peripheral CD4+CD127loCD25hiFoxP3+ Treg cells in chronic HCV infected-patients. *Virol J* **8**, 279, doi:10.1186/1743-422X-8-279 (2011).

77 Kakumu, S. *et al.* Production of interleukins 10 and 12 by peripheral blood mononuclear cells (PBMC) in chronic hepatitis C virus (HCV) infection. *Clin Exp Immunol* **108**, 138-143 (1997).

78 Lohmann, V. *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* **285**, 110-113 (1999).

79 Heller, T. *et al.* An in vitro model of hepatitis C virion production. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 2579-2583, doi:10.1073/pnas.0409666102 (2005).

80 Lindenbach, B. D. *et al.* Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 3805-3809, doi:10.1073/pnas.0511218103 (2006).

81 Bukh, J. *et al.* Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 14416-14421, doi:10.1073/pnas.212532699 (2002).

82 Kato, T. *et al.* Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. *Gastroenterology* **125**, 1808-1817 (2003).

83 Blight, K. J., McKeating, J. A., Marcotrigiano, J. & Rice, C. M. Efficient replication of hepatitis C virus genotype 1a RNAs in cell culture. *Journal of virology* **77**, 3181-3190 (2003).

84 Murray, E. M. *et al.* Persistent replication of hepatitis C virus replicons expressing the beta-lactamase reporter in subpopulations of highly permissive Huh7 cells. *Journal of virology* **77**, 2928-2935 (2003).

85 Ilan, E. *et al.* The hepatitis C virus (HCV)-Trimera mouse: a model for evaluation of agents against HCV. *J Infect Dis* **185**, 153-161, doi:10.1086/338266 (2002).

86 Mercer, D. F. *et al.* Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**, 927-933, doi:10.1038/90968 (2001).

87 He, L. & Hannon, G. J. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* **5**, 522-531, doi:10.1038/nrg1379 (2004).

88 Bagga, S. *et al.* Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* **122**, 553-563, doi:10.1016/j.cell.2005.07.031 (2005).

89 Bartel, D. P. MicroRNAs: target recognition and regulatory functions. *Cell* **136**, 215-233, doi:10.1016/j.cell.2009.01.002 (2009).

90 Xu, P., Vernooy, S. Y., Guo, M. & Hay, B. A. The Drosophila microRNA Mir-14 suppresses cell death and is required for normal fat metabolism. *Curr Biol* **13**, 790-795 (2003).

91 Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B. & Cohen, S. M. bantam encodes a developmentally regulated microRNA that controls cell proliferation and regulates the proapoptotic gene hid in Drosophila. *Cell* **113**, 25-36 (2003).

92 Lagos-Quintana, M. *et al.* Identification of tissue-specific microRNAs from mouse. *Curr Biol* **12**, 735-739 (2002).

93 Esau, C. *et al.* miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. *Cell Metab* **3**, 87-98, doi:10.1016/j.cmet.2006.01.005 (2006).

94 Krutzfeldt, J. *et al.* Silencing of microRNAs in vivo with 'antagomirs'. *Nature* **438**, 685-689, doi:10.1038/nature04303 (2005).

95 Jopling, C. L., Schutz, S. & Sarnow, P. Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. *Cell Host Microbe* **4**, 77-85, doi:10.1016/j.chom.2008.05.013 (2008).

96 Jopling, C. L., Yi, M., Lancaster, A. M., Lemon, S. M. & Sarnow, P. Modulation of hepatitis C virus RNA abundance by a liver-specific MicroRNA. *Science* **309**, 1577-1581, doi:10.1126/science.1113329 (2005).

97 Lanford, R. E. *et al.* Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. *Science* **327**, 198-201, doi:10.1126/science.1178178 (2010).

98 Sarasin-Filipowicz, M., Krol, J., Markiewicz, I., Heim, M. H. & Filipowicz, W. Decreased levels of microRNA miR-122 in individuals with hepatitis C responding poorly to interferon therapy. *Nat Med* **15**, 31-33, doi:10.1038/nm.1902 (2009).

99 Helbig, K. J., Lau, D. T., Semendric, L., Harley, H. A. & Beard, M. R. Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector. *Hepatology* **42**, 702-710, doi:10.1002/hep.20844 (2005).

100 Su, A. I. *et al.* Genomic analysis of the host response to hepatitis C virus infection. *Proceedings of the National Academy of Sciences of the United States of America* **99**, 15669-15674, doi:10.1073/pnas.202608199 (2002).

101 Suppiah, V. *et al.* IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. *Nature genetics* **41**, 1100-1104, doi:10.1038/ng.447 (2009).

102 Ge, D. *et al.* Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* **461**, 399-401, doi:10.1038/nature08309 (2009).

103 Tanaka, Y. *et al.* Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nature genetics* **41**, 1105-1109, doi:10.1038/ng.449 (2009).

104 Thomas, D. L. *et al.* Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. *Nature* **461**, 798-801, doi:10.1038/nature08463 (2009).

105 May, T. *et al.* Synthetic gene regulation circuits for control of cell expansion. *Tissue Eng Part A* **16**, 441-452, doi:10.1089/ten.TEA.2009.0184 (2010).

106 May, T., Hauser, H. & Wirth, D. In vitro expansion of tissue cells by conditional proliferation. *Methods Mol Med* **140**, 1-15 (2007).

107 Scherr, M. *et al.* Lentivirus-mediated antagomir expression for specific inhibition of miRNA function. *Nucleic acids research* **35**, e149, doi:10.1093/nar/gkm971 (2007).

108 Lee, Y. B., Glover, C. P., Cosgrave, A. S., Bienemann, A. & Uney, J. B. Optimizing regulatable gene expression using adenoviral vectors. *Exp Physiol* **90**, 33-37, doi:10.1113/expphysiol.2004.028209 (2005).

109 Donello, J. E., Loeb, J. E. & Hope, T. J. Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *Journal of virology* **72**, 5085-5092 (1998).

110 Kato, T. *et al.* Production of infectious hepatitis C virus of various genotypes in cell cultures. *Journal of virology* **81**, 4405-4411, doi:10.1128/JVI.02334-06 (2007).
111 Pfeffer, L. M., Madey, M. A., Riely, C. A. & Fleckenstein, J. F. The induction of type I interferon production in hepatitis C-infected patients. *J Interferon Cytokine Res* **29**, 299-306, doi:10.1089/jir.2008.0092 (2009).
112 Ghislain, J. J., Wong, T., Nguyen, M. & Fish, E. N. The interferon-inducible Stat2:Stat1 heterodimer preferentially binds in vitro to a consensus element found in the promoters of a subset of interferon-stimulated genes. *J Interferon Cytokine Res* **21**, 379-388, doi:10.1089/107999001750277853 (2001).
113 Lin, W. *et al.* Hepatitis C virus expression suppresses interferon signaling by degrading STAT1. *Gastroenterology* **128**, 1034-1041 (2005).
114 Lin, W. *et al.* Hepatitis C virus core protein blocks interferon signaling by interaction with the STAT1 SH2 domain. *Journal of virology* **80**, 9226-9235, doi:10.1128/JVI.00459-06 (2006).
115 Wietzke-Braun, P. *et al.* Interferon regulatory factor-1 promoter polymorphism and the outcome of hepatitis C virus infection. *Eur J Gastroenterol Hepatol* **18**, 991-997, doi:10.1097/01.meg.0000224478.89545.76 (2006).
116 Zekri, A. R. *et al.* Disease progression from chronic hepatitis C to cirrhosis and hepatocellular carcinoma is associated with repression of interferon regulatory factor-1. *Eur J Gastroenterol Hepatol* **22**, 450-456, doi:10.1097/MEG.0b013e3283329d00 (2010).
117 Saito, T., Owen, D. M., Jiang, F., Marcotrigiano, J. & Gale, M., Jr. Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA. *Nature* **454**, 523-527, doi:10.1038/nature07106 (2008).
118 Sumpter, R., Jr. *et al.* Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. *Journal of virology* **79**, 2689-2699, doi:10.1128/JVI.79.5.2689-2699.2005 (2005).
119 Pichlmair, A. *et al.* RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science* **314**, 997-1001, doi:10.1126/science.1132998 (2006).
120 Hornung, V. *et al.* 5'-Triphosphate RNA is the ligand for RIG-I. *Science* **314**, 994-997, doi:10.1126/science.1132505 (2006).
121 Pulverer, J. E. *et al.* Temporal and spatial resolution of type I and III interferon responses in vivo. *Journal of virology* **84**, 8626-8638, doi:10.1128/JVI.00303-10 (2010).
122 Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* **413**, 732-738, doi:10.1038/35099560 (2001).
123 Dixit, E. *et al.* Peroxisomes are signaling platforms for antiviral innate immunity. *Cell* **141**, 668-681, doi:10.1016/j.cell.2010.04.018 (2010).
124 Li, Y. *et al.* Morphine inhibits intrahepatic interferon- alpha expression and enhances complete hepatitis C virus replication. *J Infect Dis* **196**, 719-730, doi:10.1086/520093 (2007).
125 Ye, L. *et al.* Methamphetamine enhances Hepatitis C virus replication in human hepatocytes. *J Viral Hepat* **15**, 261-270, doi:10.1111/j.1365-2893.2007.00940.x (2008).
126 Raychoudhuri, A. *et al.* Hepatitis C virus infection impairs IRF-7 translocation and Alpha interferon synthesis in immortalized human hepatocytes. *Journal of virology* **84**, 10991-10998, doi:10.1128/JVI.00900-10 (2010).

127 Fougere-Deschatrette, C. *et al.* Plasticity of hepatic cell differentiation: bipotential adult mouse liver clonal cell lines competent to differentiate in vitro and in vivo. *Stem cells* **24**, 2098-2109, doi:10.1634/stemcells.2006-0009 (2006).

128 Ank, N. *et al.* An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. *Journal of immunology* **180**, 2474-2485 (2008).

129 Marcello, T. *et al.* Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. *Gastroenterology* **131**, 1887-1898, doi:10.1053/j.gastro.2006.09.052 (2006).

130 McHutchison, J. G. *et al.* Hepatic HCV RNA before and after treatment with interferon alone or combined with ribavirin. *Hepatology* **35**, 688-693, doi:10.1053/jhep.2002.31870 (2002).

131 Melia, M. T. *et al.* Racial differences in hepatitis C treatment eligibility. *Hepatology* **54**, 70-78, doi:10.1002/hep.24358 (2011).

132 Wang, S. *et al.* Viperin inhibits hepatitis C virus replication by interfering with binding of NS5A to host protein hVAP-33. *The Journal of general virology* **93**, 83-92, doi:10.1099/vir.0.033860-0 (2012).

133 Wang, N. *et al.* Toll-like receptor 3 mediates establishment of an antiviral state against hepatitis C virus in hepatoma cells. *Journal of virology* **83**, 9824-9834, doi:10.1128/JVI.01125-09 (2009).

134 Lohmann, V. in *HEPATITIS C*
Methods in Molecular Biology Vol. 510 145-163 (2009).

135 van der Eijk, A. A., Vrolijk, J. M. & Haagmans, B. L. Antibodies neutralizing peginterferon alfa during retreatment of hepatitis C. *The New England journal of medicine* **354**, 1323-1324, doi:10.1056/NEJMc052880 (2006).

136 Barnes, B. J., Moore, P. A. & Pitha, P. M. Virus-specific activation of a novel interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes. *The Journal of biological chemistry* **276**, 23382-23390, doi:10.1074/jbc.M101216200 (2001).

137 Arnaud, N. *et al.* Hepatitis C virus controls interferon production through PKR activation. *PloS one* **5**, e10575, doi:10.1371/journal.pone.0010575 (2010).

138 Chen, C. W. *et al.* The double-stranded RNA-activated kinase, PKR, can phosphorylate hepatitis D virus small delta antigen at functional serine and threonine residues. *The Journal of biological chemistry* **277**, 33058-33067, doi:10.1074/jbc.M200613200 (2002).

139 Garaigorta, U. & Chisari, F. V. Hepatitis C virus blocks interferon effector function by inducing protein kinase R phosphorylation. *Cell Host Microbe* **6**, 513-522, doi:10.1016/j.chom.2009.11.004 (2009).

140 Duong, F. H., Filipowicz, M., Tripodi, M., La Monica, N. & Heim, M. H. Hepatitis C virus inhibits interferon signaling through up-regulation of protein phosphatase 2A. *Gastroenterology* **126**, 263-277 (2004).

141 Robek, M. D., Boyd, B. S. & Chisari, F. V. Lambda interferon inhibits hepatitis B and C virus replication. *Journal of virology* **79**, 3851-3854, doi:10.1128/JVI.79.6.3851-3854.2005 (2005).

142 Doyle, S. E. *et al.* Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. *Hepatology* **44**, 896-906, doi:10.1002/hep.21312 (2006).

143 Kotenko, S. V. *et al.* IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nature immunology* **4**, 69-77, doi:10.1038/ni875 (2003).

144 Sommereyns, C., Paul, S., Staeheli, P. & Michiels, T. IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS pathogens* **4**, e1000017, doi:10.1371/journal.ppat.1000017 (2008).

145 Francois-Newton, V. *et al.* USP18-based negative feedback control is induced by type I and type III interferons and specifically inactivates interferon alpha response. *PloS one* **6**, e22200, doi:10.1371/journal.pone.0022200 (2011).

146 Stiffler, J. D. *et al.* Focal distribution of hepatitis C virus RNA in infected livers. *PloS one* **4**, e6661, doi:10.1371/journal.pone.0006661 (2009).

147 Onoguchi, K. *et al.* Viral infections activate types I and III interferon genes through a common mechanism. *The Journal of biological chemistry* **282**, 7576-7581, doi:10.1074/jbc.M608618200 (2007).

6. Appendix

qPCR primers-mouse

Primer Name	Primer Sequence
Mu.gbp1-fwd	cagggtagacaatgggcagt
Mu.gbp1-rev	cacagtaggctggagcatga
Mu.mx2-fwd	tcaccagagtgcaagtgagg
Mu.mx2-rev	cattctccctctgccacatt
Mu.Rsad2-fwd	gtcctgtttggtgcctgaat
Mu.Rsad2-rev	gccacgcttcagaaacatct
Mu.isg20-fwd	taagcgcctgctacacaaga
Mu.isg20-rev	gcagcttctaaccctggatg
Mu.usp18-fwd	aaggaccagatcacggacac
Mu.usp18-rev	catcctccagggttttcaga
Mu.irf9-fwd	accacggaaccagaaatcac
Mu.irf9-rev	gttgcagttgctgttgctgt
Mu.beta actin-fwd	tggaatcctgtggcatccatgaaa
Mu.beta actin-rev	taaaacgcagctcagtaacagtccg
Mu.Isg15-fwd	gagaggcagcgaactcatct
Mu.Isg15-rev	cttcagctctgacaccgaca
Mu.Ifit2-fwd	gcgtgaagaaggtgaagagg
Mu.Ifit2-rev	gcaggtaggcattgtttggt
Mu.Viperin-fwd	tgcttggtgcctgaatctaa
Mu.Viperin-rev	tttcctcctcgcttcagaaa
Mu.Usp18-fwd	cagaccctgacaatccacct
Mu.Usp18-rev	agctcatactgccctccaga
Mu.Isg20-fwd	gcacaagagcatccagaaca
Mu.Isg20-rev	aagccgaaagcctctagtcc
Mu.Mx2-fwd	aagcagtatcgaggcaagga
Mu.Mx2-rev	tcgtgctctgaacagtttgg
Mu.IRF9-fwd	aggtccagctgtctggaaga
Mu.IRF9-rev	actgtgctgtcgctttgatg
Mu.IFNbeta-fwd	cattacctgaaggccaagga
Mu.IFNbeta-rev	cagcatctgctggttgaaga
Mu.IFNalfa4-fwd	ccagttccagaaggctcaag
Mu.IFNalfa4-rev	agtctcttccaccccaacct
Mu.Oas-fwd	caagctcaagagcctcatcc
Mu.Oas-rev	tgggctgtgttgaaatgtgt

qPCR primers-human

Primer Name	Primer Sequence
hAPOBEC3G-fwd	ggatgaagcctcacttcaga
hAPOBEC3G-rev	atacacctggcctcgaaag
h.IFIT3-fwd	cagaactgcagggaaacagcc
h.IFIT4-rev	gccaacaagttgtacattgtagc
h.IFI-6-fwd	ctcgctgatgagctggtc
h.IFI-7-rev	ggccaagaaggaagaagagg
h.ISG15-fwd	gagaggcagcgaactcatct
h.ISG15-rev	cttcagctctgacaccgaca
h.IFIT2/ISG54-fwd	gcgtgaagaaggtgaagagg
h.IFIT2/ISG54-rev	gcaggtaggcattgtttggt
h.IP10/CXCL10-fwd	aggaacctccagtctcagca
h.IP10/CXCL10-rev	caaaattggcttgcaggaat
h.Viperin-fwd	tgcttggtgcctgaatctaa
h.Viperin-rev	tttcctcctcgcttcagaaa
h.USP18-fwd	cagaccctgacaatccacct
h.USP18-rev	agctcatactgccctccaga
h.ISG20-fwd	gcacaagagcatccagaaca
h.ISG20-rev	aagccgaaagcctctagtcc
h.Mx2-fwd	aagcagtatcgaggcaagga
h.Mx2-rev	tcgtgctctgaacagtttgg
h.IRF9-fwd	aggtccagctgtctggaaga
h.IRF9-rev	actgtgctgtcgctttgatg
h.IFN beta1-fwd	cattacctgaaggccaagga
h.IFN beta1-rev	cagcatctgctggttgaaga
h.GBP1-fwd	ggtccagttgctgaaagagc
h.GBP1-rev	tgacaggaaggctctggtct
h.IFN-alpha 4-fwd	ccagttccagaaggctcaag
h.IFN-alpha 4-rev	agtctcttccaccccaacct
h.OAS-fwd	caagctcaagagcctcatcc
h.OAS-rev	tgggctgtgttgaaatgtgt

h.IRF7 -fwd	tacgggtgggcagtagagac
h.IRF7 -rev	ggcccttgtacatgatggtc
h.IFI6-fwd	accttccctgagagccatct
h.IFI7-rev	atctgaggagtgtggggatg
h.PKR -fwd	caaggggaaaacgaaactga
h.PKR -rev	attctgaagaccgccagaga
Other primers	
HProm_BsaB1fwd	AATTCCTGCGATGCAGATCCGG
HProm_Agerev	AATTACCGGTGGATCCTCGCGAGTTTAAACCAG CTTAGCTTGGCAGAAC
HPromtTA_Pmel	AATTGTTTAAACCACGAGGCCCTTTCGTC
HPromtTA_BamHl	TTAATCGCGACTATAGTGAGTCGTATTACCTGC AGGGGCCGCGGAGGCTGG
Hprom5U_BamH1	AATTGGATCCACCTGCCCCTAATAGGGG
Hprom5U_Age1	AATTCACCGGTTCCGCAGACCAC
Hsel_Age	AATTGAACCGGTGAGTACACCG
Hsel_Not	AATTGCGCGGCCGCTTACAATTTG
miR122for	AATTCCCTTAGCAGAGCTGTGGAGTGTGACAAT GGTGTTTGTGTCTAAACTATCAAACGCCATTAT CACACTAAATAGCTACTGCTAGGCG
miR122rev	GATCCGCCTAGCAGTAGCTATTTAGTGTGATAA TGGCGTTTGATAGTTTAGACACAAACACCATTG TCACACTCCACAGCTCTGCTAAGGG

Plasmid vectors

- JFH-1prom3 and JFH-1ΔGDDprom3

The plasmid was designed to make the subgenoimc replicon pFKi389 NS3JFH1 or the pFKi389 NS3JFH1ΔGDD encoding the genotype2a strain into a tet regulatable system. To this end, an intermediate plasmid cloned HCVprom3.

The helper vector was cloned by performing a PCR on the pFKi389 NS3 JFH1 vector to amplify a 321bp region before the T7 promter using primers HProm_BsaB1 and HProm_Age. This vector was the used to embed a PCR amplified pTBC-1 vector carrying the Tet promoter using primers HPromtTA_PmeI and HPromtTA_BamHI. Following this,

PCR derived 5’ NTR using primers HProm5U_BamHI and HProm5U_Age from the pFKi389 NS3 JFH1 vector was embedded. This vector is referred to as the HCV prom3. PmeI and AgeI restricted HCV prom3 vector was ligated to a PmeI and AgeI restricted pFKi389 NS3 JFH1 or pFKi389 NS3JFH1ΔGDD.

- JFH-1pTight and JFH-1ΔGDDpTight

The helper vector was cloned by performing a PCR on the pFKi389 NS3JFH1 or the pFKi389 NS3JFH1ΔGDD vector to amplify a 321bp region before the T7 promter. This vector was the used to embed a PCR amplified pTRE-Tight vector carrying the Tet promoter. Following this, PCR derived 5’ NTR from the pFKi389 NS3 JFH1 vector was embedded. This vector is referred to as the HCV pTight. PmeI and AgeI restricted HCV prom3 vector was ligated to a PmeI and AgeI restricted pFKi389 NS3JFH1 or the pFKi389 NS3JFH1ΔGDD.

- SIEW-miR122

Oligos coding for miR122-f and miR122-r were phosphorylated and hybridized with each other. Plasmid pSuperΔEΔB was restricted with ECoRI and BamHI and ligated to the annealed oligos. The miR122 and the surrounding miR-30 was PCR amplified from the resulting vector pSuperΔEΔB-miR122 with BglII flanking primers and ligated into BamHI restricted lentiviral vector SIEW.

6.1 List of Figures

6.2 List of Tables

6.3 Abbreviations

α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
κ	kappa
λ	lambda
μ	micro
A	adenosine
APC	antigen presenting cell
ATP	adenosintriphoshate
bp	base pair
BSA	bovine serum albumin
C	cytosine
CARD	caspase recruitment domain
cDNA	complementary DNA
CMV	cytomegalovirus
Da	dalton
DAA	direct-acting antiviral
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleicacid
dNTP	deoxyribonucleotidetriphosphate
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
eGFP	enhanced GFP
eIF-2	eukaryotic initiation factor-2
FBS	fetal bovine serum
F-luc	Firefly luciferase
G	guanidine
G418	aminoglycoside-2´-deoxystreptin
GFP	green fluorescent protein
GMP	guanosine monophosphate
GTPase	guanosine triphosphatase
h	hours
HAU	haem agglutinating unit
HCC	hepato cellular carcinoma
HCV	hepatitis-C virus
HMG	high-mobility group
ICSBP	IFN consensus sequence binding protein
IFN	interferon
IFNAR	interferon-α/β receptor
IKK	inhibitor of NF-κB kinase
IL	interleukin
IPS-1	IFN-β promoter stimulator
IRF	IFN regulatory factor
IRFE	IRF-element

ISG	IFN stimulated gene
ISGF	IFN stimulated gene factor
ISRE	IFN stimulated response elements
JAK	Janus activated kinase
JFH-1	Japanese fulminant hepatitis-1
Kn	kanamycin
LGP-2	laboratory of genetics and physiology-2
LPS	lipopolysaccharide
Luc	luciferase
m	milli
M	molarity
Mda5	melanoma-differentiation-associated gene 5
MEF	mouse embryonic fibroblast
MHC	major histocompatibility complex
min	minute
mRNA	messenger RNA
miR-122	MicroRNA-122
miRISC	miRNA-induced silencing complex
MOI	multiplicity of infection
Mx	myxovirus resistance gene
MyD88	myeloid differentiation factor gene 88
n	nano
NANB	non A non B
NDV	Newcastle disease virus
nptII	neomycin phosphotransferase
NF-κB	nuclear factor κB
NTR	non-translated region
OAS	oligoadenylate synthetase
OD	optical density
ORF	open reading frame
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGK	phosphoglycerate kinase
PKR	protein kinase R
PLB	passive lysis buffer
poly I:C	polyinosinic-polycytidylic acid
PRD	positive regulatory domain
PRR	pathogen recognition receptor
qPCR	quantitative PCR
RIG-I	retinoic-acid-inducible gene I
RLH	RIG-I-like helicases
RLU	relative light units
RNA	ribonucleic acid
RNase	ribonuclease
Rt-PCR	reverse transcriptase PCR
s	second
SDS	sodium dodecyl sulfate
SOCS	suppressor of cytokine signalling
SNP	single nucleotide polymorphism
STAT	signal transducer and activator of transcription

STING	stimulator of IFN genes
SR-B1	scavenger receptor class B type I
SV40	simian virus 40
T	thymidine
TBK-1	TANK-binding kinase 1
Tet	tetracycline
TIR	Toll/interleukin-1 receptor homology
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TRAF3	TNF-receptor associated factor 3
TRIF	TIR-domain containing adaptor inducing IFN-β
TRIM25	tripartite-motif containing protein 25
Tris	tris-hydroxymethyl-aminomethan
Tyk2	tyrosin kinase 2
U	unit
USP-18	ubiquitin-specific peptidase 18
VSV	vesicular stomatitis virus
WT	wild type

Latinisms are written in italics.

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